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ARTICLE

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DT diaphorase exists as a dimer-tetramer equilibrium in solution

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Abstract The quaternary behaviour of DT diaphorase in solution has been investigated by hydrodynamics under a range of conditions. At neutral pH DT diaphorase is shown to exist as a tightly-associated homodimer in a dimer-tetramer equilibrium. Concentrations of the chaotropic agent potassium thiocyanate (KSCN) of greater than 200 mM result in irreversible loss of the FAD cofactor and denaturation of the homodimer though this agent appears to be ineffective in disrupting intermolecular association. These data conform to a model in which under extreme dissociation conditions, the folded dimer is in equilibrium with the unfolded monomer and are consistent with evidence from the X-ray structure and proposed catalytic mechanism where both monomers are catalytically interdependent.

Key words Flavoprotein · FAD · Dehydrogenase · Self-association · Analytical ultracentrifugation

Introduction

DT diaphorase (NAD(P)H dehydrogenase (quinone) (EC 1.6.99.2)) is a widely distributed flavoprotein which promotes the direct aerobic reduction of quinones, quinoneimines and azo dyes (Ernster 1987). It can utilise either NADH or NADPH and has the unique ability to transfer two electrons directly to its substrate (Iyanagi and Yamazaki 1970). This reaction protects cells from the damaging effects of reactive oxygen species and free radicals generated as a result of the one-electron reductions catalysed by cytochrome P450's (for a review see Ernster 1987). It therefore has an important protective function against carcinogenicity and cytotoxicity. However, the role of this enzyme is not always protective and it has also been implicated in the activation of some quinone compounds and certain nitro-compounds to cytotoxic forms (reviewed in Riley and Workman 1992). DT diaphorase activates some important chemotherapeutic quinones including the mitomycins (Ross et al. 1993). This property is of current interest in the development of novel approaches to enzyme-targeted chemotherapy (Knox 1993).

DT diaphorase is a dimer of identical subunits, each comprising 273 amino acids (Robertson et al. 1986; Bayney et al. 1987; Haniu et al. 1988). Each monomer contains a non-covalently bound FAD prosthetic group which is essential for catalytic activity. A two-domain structure for the monomer proposed on the basis of proteolytic digestion studies (Chen et al. 1994) has been confirmed by the recent X-ray structure of the rat enzyme (Li et al. 1995). This reveals that each subunit contains a catalytic domain possessing a twisted central parallel beta sheet, a fold resembling that of many other flavoproteins. The structure has now shed light on the nature of the obligatory 2-electron reduction mechanism and an explanation has been proposed based on the dimeric status of the enzyme which was hitherto uncertain. The proposed mechanism for the 2-electron reduction would require DT diaphorase to behave as a dimer in solution. Despite the early reports of the existence of the rat enzyme as a dimer (Huang et al. 1979), in the case of the Walker tumour enzyme, there was strong evidence to suggest its behaviour as a monomer in solution (Knox et al. 1988). Furthermore, the human form of the enzyme gave a very low molecular weight when determined by size-exclusion chromatography (Boland et al. 1991). DT diaphorase contains a single cysteine residue per subunit which is not essential for activity (Ma et al. 1992). Also, S-alkylation of this residue under non-reducing conditions has shown that it is not involved in disulphide linkage (Haniu et al. 1988). To investigate the quaternary nature of DT diaphorase and understand further the mechanism of the two-electron reduction in solution we have carried out molecular hydrodynamic studies and

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observed the dissociation and unfolding under a range of experimental conditions. Analytical enzyme assays were used to monitor the enzyme activity.

Materials and methods

Expression and purification of recombinant DT diaphorase

A fragment containing rat full-length (NMOR1) cDNA (Robertson et al. 1986) was subcloned into plasmid pET11d (Novagen Inc.) under the control of the bacteriophage T7 *lac* promoter, and expressed in BL21(DE3)pLysS cells. Protein was expressed by induction with 1 mM IPTG. The bacterial pellet was harvested by centrifugation and resuspended in 50 mM Tris, pH 7.5 containing 10 mM $MgCl_2$, and 1 mM aprotinin. The bacterial cell walls were disrupted by sonication. After centrifugation at 12,000 g the cell lysate was treated with 1% polyethylene imine to remove nucleic acids before chromatography on a Hi-load 16/60 Superose 12 (prep-grade) column. Fractions were assayed for DT diaphorase activity and the active fractions chromatographed by anion exchange on Q-Sepharose Hi-load.

DT diaphorase was eluted by a salt gradient in 10 mM sodium phosphate, pH 7.0 containing 1 mM PMSF, 10 mM NaCl. The protein was visualised on SDS PAGE gels stained with PAGE Blue 83. Minor impurities were finally removed by size-exclusion on Superdex-75 HR 10/30. A total yield of 15 mg of pure protein per litre of culture was obtained. DT-diaphorase was measured using the molar extinction coefficient at 451 nm in a 10 mm pathlength (6.65×10^4 cm^2/mol). The protein was concentrated for hydrodynamic and other experiments using an Amicon Centricon with a 30 k Da cut off. Where it was necessary to ensure reduction of the thiol groups 10 mM dithiothreitol was added to the buffer.

Enzyme assays

DT diaphorase activities were assayed as described previously (Knox et al. 1988) using menadione as the substrate and cytochrome c as the terminal electron acceptor. Stock concentrations of menadione (100 mM) were made up in 100% DMSO. All other reagents were made up in 10 mM sodium phosphate, pH 7.0. The assays were carried out at 37 °C.

KSCN-Induced inactivation of DT diaphorase

The enzyme (10 mg/ml) was incubated at 20 °C in sodium phosphate, pH 7.0 containing a range of 50 mM to 400 mM KSCN following the procedure of Deville-Bonne et al. 1989. The enzyme was reactivated by diluting 1:100 followed by buffer exchange in 10 mM phosphate, pH 7.0.

Sedimentation equilibrium

The degree and strength of oligomerisation of DT diaphorase was determined with sedimentation equilibrium. Firstly, runs were performed with a Beckman (Palo Alto, USA) Optima XL-A analytical ultracentrifuge (AUC) equipped with scanning absorbance optics. DT diaphorase was studied in native conditions (10 mM sodium phosphate, pH 7.0). Rotor speeds of 17,000 and 21,000 rpm were used and the temperature was maintained at 20 °C throughout. Samples of concentrations between 0.05 and 4.65 mg/ml were loaded into standard double-sector cells with a column height of 2 mm, in order to achieve good accuracy in the subsequent data analysis. The attainment of equilibrium was verified by the satisfactory overlay of scans acquired three hours apart. Once equilibrium was achieved the rotor speed was increased to 40,000 rpm so that a true optical baseline was determined, free from macromolecular solute. Scans were performed either at near-UV wavelengths (273–275 nm) for concentrations below 1.0 mg/ml or in the region of the flavin chromophore (407–473 nm) for higher concentrations. The significant oligomerisation observed upon analysis of the data resulting from these runs (Results) may have been due to covalent cross-linking between cysteines. Therefore further runs were performed as outlined above for DT diaphorase in which the thiol groups had been reduced. Samples of concentration 1.5, 3.0 and 5.0 mg/ml were loaded into double-sector cells while 6-hole Yphantis-type cells were used for samples in the concentration range 0.05–0.80 mg/ml. The level of oligomerisation was unaffected by this treatment. Therefore the sample was run in a chaotropic buffer (200 mM KSCN, 10 mM sodium phosphate, pH 7.0) in a further attempt to interfere with the association. The sample (at concentrations of 1.0, 3.0 and 5.0 mg/ml) was housed in single sector cells with wedge windows and run at a rotor speed of 17,420 rpm at 20 °C in an MSE (Crawley, UK) Mk II AUC. The equilibrium solute distribution was recorded with schlieren optics so that the significant absorbance of the KSCN did not mask the protein signal.

Sedimentation velocity

The weight-average sedimentation coefficient of DT diaphorase was determined using sedimentation velocity. Four runs were performed: two in 10 mM sodium phosphate, pH 7.0 buffer in the Beckman Optima XL-A AUC (0.3–0.7 mg/ml (273 nm); 1.29–4.65 mg/ml (453 nm); 40,000 rpm, 20 °C); two in 200 mM KSCN, 10 mM sodium phosphate, pH 7.0 buffer in the MSE Mk II AUC (10.8 mg/ml, 35,140 rpm, 20 °C; 4.0 and 7.0 mg/ml, 35,160 rpm, 21 °C respectively).

The viscosity of the two buffers used in AUC studies was determined relative to water at 20 °C with a Schott-Geräte (Hofheim, Germany) automatic viscometry system and an Ostwald capillary viscometer. An Anton Paar (Graz, Austria) DMA 0C2 precision densimeter was used

to determine the buffer density, again at 20 °C. These data (10 mM sodium phosphate, pH 7.0: $\eta_{rel} = 1.01646$, $\rho_{20} = 1.0032$ g/ml; 200 mM KSCN, 10 mM sodium phosphate, pH 7.0: $\eta_{rel} = 0.99592$, $\rho_{20} = 1.0051$ g/ml) were then used to standardise hydrodynamic parameters to conditions of water as a solvent at 20 °C. The partial specific volume ($\bar{v} = 0.747$ ml/g) was calculated from the known amino acid composition using data for the partial specific volumes of the constituent amino acids (Perkins 1986).

Results

Enzyme activity

The enzymic stability of DT diaphorase was monitored under relatively high ionic strengths of up to 500 mM KCl and also in the presence of up to 800 mM potassium thiocyanate (KSCN), a chaotropic agent known to destabilise interactions between proteins. No significant change in activity was observed with increasing ionic strength. At 200 mM KSCN 70% of the original enzymic activity was lost (see Fig. 1). This was reversed by a 100:1 dilution with sodium phosphate, pH 7.0. Concentrations of KSCN > 400 mM resulted in almost complete inactivation of the enzyme. Less than 40% of the original activity was achieved by further buffer dilution. Gel-filtration experiments revealed that incubation with > 200 mM KSCN resulted in the gradual and irreversible loss of the FAD prosthetic group which is essential for activity. Excess FAD was included in the enzyme reaction mixture for the reactivation experiments.

The best-fit model derived from analysis of the sedimentation equilibrium results (below) corresponds to a monomer mass of the homodimer. On SDS PAGE the protein runs as a single monomer of 30 kDa under both reducing and non-reducing conditions. The possibility of covalent linkage due to oxidation of the single thiol group in the monomer was excluded by including DTT in the buffers throughout the purification and during the runs. Addition of the chaotropic agent KSCN up to 200 mM does not appear to disrupt the association. However, most of the original activity is lost (although reversible) at this concentration. The FAD prosthetic group is noncovalently attached and can be reversibly removed by dialysis against 2 M potassium bromide (Shiuan Chen personal communication). At higher KSCN concentrations the enzyme is irreversibly inactivated or denatured.

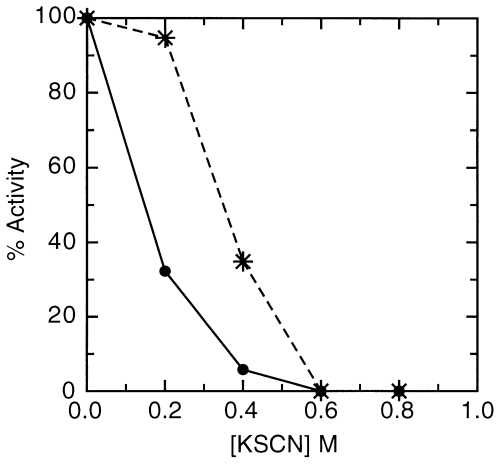

Fig. 1 Inactivation of DT diaphorase by KSCN expressed as a percentage of the original activity. The enzyme (10 mg/ml) is incubated at 20 °C for 20 h at each concentration of KSCN in 10 mM sodium phosphate, pH 7.0. Reactivation is carried out by 1:100 dilution with buffer. Inactivation (●, – – –); Reactivation (*, - - - -)

Analytical ultracentrifugation

Sedimentation equilibrium

The equilibrium solute distributions obtained with absorbance optics (Fig. 2) were firstly fitted with the equation for a single ideal species.

$$A_r = A_0 \exp[H \cdot M(r^2 - r_0^2)] + E \quad (1)$$

where A_r is the absorbance at radial position r and A_0 the absorbance at a reference position r_0; H is the constant $(1-\bar{v}\rho)\omega^2/2RT$; $\bar{v}$ is the partial specific volume of the

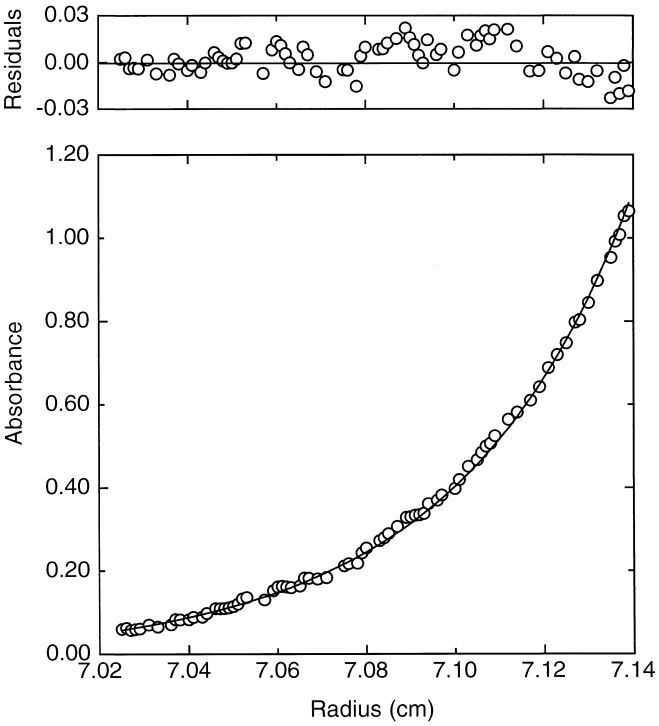

Fig. 2 Sedimentation equilibrium solute distribution for DT diaphorase at a loading concentration of 0.5 mg/ml observed at 275 nm. The data were initially fitted with the equation for a single, ideal species (Eq. (1)) but a better fit was obtained with a dimer-tetramer form of Eq. (2). The randomness of the residuals indicates the satisfactory nature of the model used. This fit is typical of all fits obtained for DT diaphorase in 10 mM sodium phosphate, pH 7.0

macromolecule; ρ is the solvent density; ω is the rotor speed (radians/s); R is the gas constant; T is the temperature (K); M is the molecular weight of the solute; E is the optical baseline offset. A good fit with this equation (as judged by the randomness and magnitude of the residuals) yields the apparent whole-cell weight average molecular weight ($M_{w,app}$). In most cases good fits were obtained for DT diaphorase. The exceptions to this trend were the very low concentration (0.05 and 0.1 mg/ml) data for DT diaphorase with reduced thiols. It is possible in these instances that the cell windows may have been contaminated by the solute which subsequently failed to distribute correctly at equilibrium. It is clear from Fig. 3 that $M_{w,app}$ greatly exceeds the monomer mass of DT diaphorase (30,817 Da according to the amino acid sequence (Chen et al. 1994); 30,819 Da measured by mass spectroscopy (Chen et al. 1994) and is in fact in excess of the mass of a dimer. The same fit was performed for DT diaphorase which had been treated to reduce thiol groups. In this case data at all concentrations used were well fitted with the model in Eq. (1). The resultant values for are plotted as a function of concentration in Fig. 3. The elevation in mass could be due either to the presence of a higher molecular weight species (perhaps an aggregate) or could result from self-association. The randomness of the residuals confirms self-association.

In order to determine the stoichiometry and strength of the association, the solute equilibrium distributions were analysed with Eq. (2) below (Kim et al. 1977) which describes a self-associating system defined by up to three association constants (Ka_2, Ka_3, Ka_4 in units of $(Absorbance)^{-(n-1)}$) for oligomers composed of n_2, n_3, n_4 monomers.

$$\begin{aligned} A_r = &\exp[\ln A_0 + H \cdot M(r^2 - r_0^2)] \\ &+ \exp[n_2 \ln A_0 + \ln Ka_2 + n_2 \cdot H \cdot M(r^2 - r_0^2)] \\ &+ \exp[n_3 \ln A_0 + \ln Ka_3 + n_3 \cdot H \cdot M(r^2 - r_0^2)] \\ &+ \exp[n_4 \ln A_0 + \ln Ka_4 + n_4 \cdot H \cdot M(r^2 - r_0^2)] + E \end{aligned} \quad (2)$$

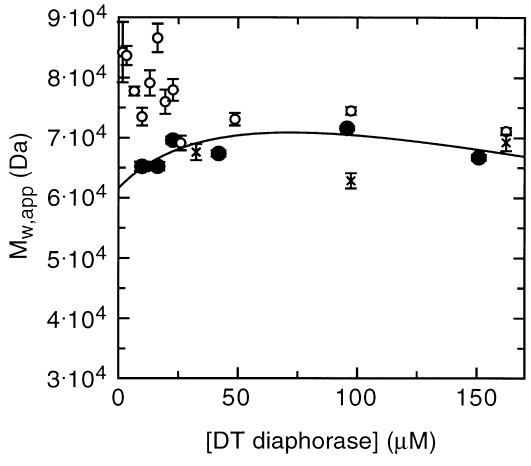

Fig. 3 Apparent weight-average molecular mass of DT diaphorase as a function of cell-loading concentration, determined with sedimentation equilibrium. (●) 10 mM sodium phosphate buffer; (○) reduced thiols; (×) 200 mM KSCN; (– – –) fit to 10 mM sodium phosphate, pH 7.0 data with Eqs. (5) and (6)

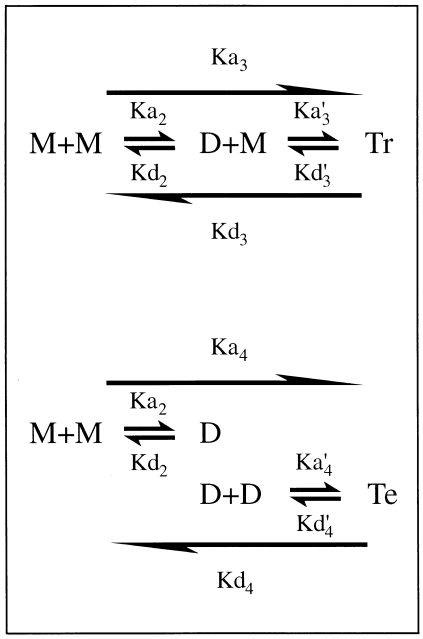

Fig. 4 Schematic of the possible equilibria represented by Eq. (2). *M*: monomer; *D*: dimer: *Tr*: trimer; *Te*: tetramer. The various Kd are defined in Results

The models tested for the oligomerisation of DT diaphorase are defined in Fig. 4. The Ka_{2-4} correspond with those defined in Eq. (2). Ka'_3 and Ka'_4 define the strength of association on going from dimer to trimer and dimer to tetramer respectively and are probably of more physical significance than Ka_3 and Ka_4 which describe the physically improbable equilibria between monomer and trimer or tetramer respectively. Ka'_3 and Ka'_4 are related to Ka_2, Ka_3 and Ka_4 as follows

$$Ka'_3 = \frac{Ka_3}{Ka_2} \quad (3a)$$

$$Ka'_4 = \frac{Ka_4}{(Ka_2)^2} \quad (3b)$$

In order to convert the units of Ka from $(Absorbance)^{-(n-1)}$ to $M^{-(n-1)}$ the following equation was used (McRorie and Voelker 1993)

$$Ka_n(c) = Ka_n(a) \cdot \frac{(\varepsilon l)^{n-1}}{n} \quad (4)$$

where ε is the molar extinction coefficient at the wavelength at which the equilibrium solute distribution was acquired; l is the pathlength of the ultracentrifuge cell (in this case 1.2 cm) and n is the number of monomers in the oligomer. For the artificial monomer-dimer model the dissociation constant was taken as the inverse of Ka.

None of the data acquired in 10 mM sodium phosphate (with either reduced or non-reduced thiols) could be fitted satisfactorily with monomer-dimer, monomer-trimer or monomer-tetramer models. It was possible to fit some of the data-sets with monomer-dimer-trimer and monomer-

dimer-tetramer models although the fits were generally rather poor. But by far the most satisfactory results were obtained by setting the monomer mass to twice its actual value (i.e. 61,636 Da instead of 30,818 Da) and performing a fit for monomer-dimer with real monomer present in such small amounts as to be invisible to the analysis. A typical fit is shown in Fig. 2. The results of this analysis are presented in Fig. 5.

The data are divided into those that result from 10 mM phosphate experiments and those from DT diaphorase in which the thiols had been reduced. The reduced thiol sample data cannot be representative of the same dimer-tetramer equilibrium and are probably spurious, as discussed earlier. The apparent Kd values for the native data can however be extrapolated to infinite dilution where a very approximate Kd of 200 μM is obtained (if the stray 23 μM data point is omitted, otherwise the extrapolation is poorly defined and yields a Kd of about 50 μM). The decrease in apparent Kd with increasing concentration seen in figure 5 is symptomatic of a solute with a finite second virial coefficient (B).

The dimer-tetramer equilibrium is represented by the equation for monomer-dimer dissociation

$$M_w = \frac{M_2}{C}\left[2C + \left(K_d - \sqrt{\{K_d^2 + 8CK_d\}}\ \ 4\right)\right] \qquad (5)$$

where the weight-average mass (M_w) is related to the 'monomer' mass (M_2 = 61,636 Da) through the molar loading concentration (C) and Kd. Then the concentration-dependent depression of apparent weight-average mass due to thermodynamic non-ideality is represented by Eq. (6).

$$M_{w,app} = \frac{M_w}{1 + 2BM_w c} \qquad (6)$$

The fit to Eqs. (5) and (6) (achieved by non-linear least squares fitting) in Fig. 3 is convincing and gives $2BM_2$ = 11 (±0.5) ml/g; Kd = 247 (±16) μM. Interestingly, this finding is not in conflict with the approximate Kd estimated from Fig. 5. Nor is the factor $2BM_2$ particularly exceptional. Neglecting contributions to non-ideality from macromolecular surface charge, if the dimer of DT diaphorase was a perfect sphere it would have $2BM_2$ = 6 ml/g. Therefore it seems likely that the dimer and tetramer of DT diaphorase are slightly elongated.

Schlieren traces recorded for DT diaphorase in chaotropic solvent (Fig. 6) were analysed on a digitising pad connected to an Apple II microcomputer. In-house purpose-written software generates a plot of log ((1/r) (dn/dr)) as a function of r^2 (where r is the radial position and n is the refractive index), the gradient of which yields the whole-cell z-average mass. This is plotted as a function of concentration in Fig. 3. There is very little difference between these data and those acquired for DT diaphorase in 10 mM sodium phosphate, indicating that the chaotropic agent appears to be ineffective in disrupting the apparent intermolecular association. Furthermore, the comparability of M_w and M_z is further evidence of a self-associating system as opposed to the presence of polydisperse species (Creeth and Pain 1967).

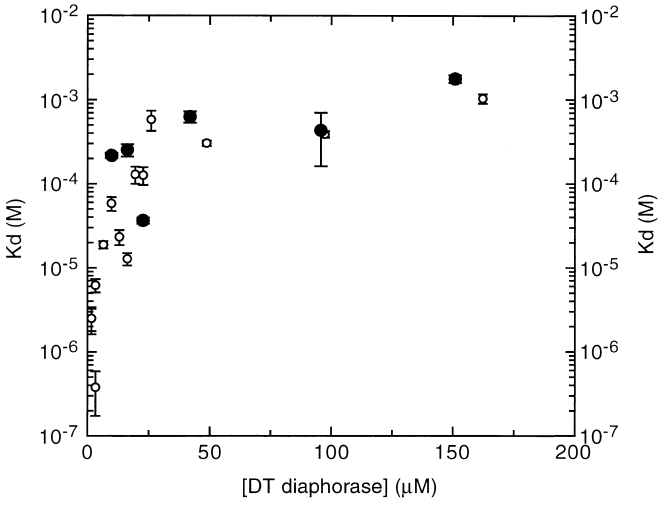

Fig. 5 The apparent dissociation constant for a dimer-tetramer model of DT diaphorase as a function of loading concentration. (●) 10 mM sodium phosphate, pH 7.0 buffer; (○) with reduced thiols

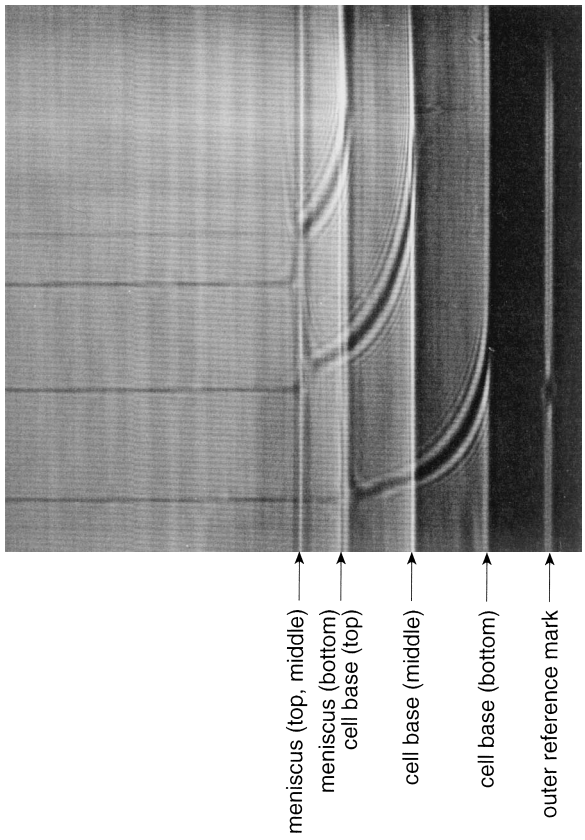

Fig. 6 The equilibrium solute distribution of DT diaphorase in 10 mM sodium phosphate, 200 mM KSCN, pH 7.0 recorded with schlieren optics. The rotor speed as 17,420 rpm and the temperature 20 °C. Loading concentrations were: (*top*) 5.0 mg/ml; (*middle*) 3.0 mg/ml; (*bottom*) 1.0 mg/ml

Sedimentation velocity

A typical set of sedimentation velocity traces for DT diaphorase in 10 mM sodium phosphate, pH 7.0 is shown in Fig. 7. Their smooth, sigmoidal character reveals that the macromolecular system consists either of a single, homogeneous species, a number of species all with very similar sedimentation coefficients or a monomer-dimer equilibrium (Gilbert and Gilbert 1973). These traces were analysed with in-house software which calculates the apparent, weight average sedimentation coefficient ($s^c_{20,b}$) from the rate of movement of the midpoint of the sedimenting boundary. These data were subsequently corrected for radial dilution effects and for the density and viscosity of buffer to yield $s^c_{20,w}$ which is plotted as a function of solute concentration in Fig. 8. Extrapolation to infinite dilution gives $s^0_{20,w} = 4.47$ S with a sedimentation velocity regression coefficient of $k_s = -35.2$ ml/g. This negative coefficient implies self-association within the macromolecular system (Gilbert and Gilbert 1973). In this case the linear extrapolation performed is inadequate and in order to determine the true value for $s^0_{20,w}$ more information is needed. So it is likely that if DT diaphorase exists in a dimer-tetramer equilibrium, the sedimentation coefficient of the dimer is less than 4.47 S.

A very basic analysis of this sedimentation coefficient makes it clear that the sedimenting system must consist of the dimeric form of DT diaphorase at least. Knowing that the frictional coefficient (f) is defined by the Stokes equation as

$$f = 6\pi\eta R \qquad (7)$$

(where R is the radius of the hydrodynamic particle $(=((3M(\bar{v}+\delta v_1))/(4\pi N_A))^{1/3}$ where v_1 is the specific volume of solvent, δ the hydration and N_A is Avogadro's number) and η is the solvent viscosity) and that this is manifest as the sedimentation coefficient (s)

$$s = (M(1-\bar{v}\rho))/N_A f \qquad (8)$$

it is possible to assess the suitability of simple spherical representations of monomer, dimer and tetramer. Thus monomeric DT diaphorase would have $s^0_{20,w} = 3.25$ S if it were an anhydrous sphere. With a moderate hydration of 0.40 g solvent/g protein this decreases to $s^0_{20,w} = 2.82$ S. Clearly, from the data in Fig. 8, DT diaphorase does not sediment as a monomer. The anhydrous spherical dimer then has $s^0_{20,w} = 5.22$ S and, in order to reproduce the linear extrapolation experimental sedimentation coefficient, would need to be hydrated to a level of 0.44 g solvent/g protein which is not unreasonable, considering the severity of the assumption made with respect to perfect sphericity. That the weight-average sedimentation coefficient increases with concentration in Fig. 8 is indicative of gradual association. This must be a dimer-tetramer equilibrium otherwise the absorbance traces in Fig. 7 would not be smooth and sigmoidal.

Schlieren records of the sedimentation of DT diaphorase in chaotropic solvent (Fig. 9) were analysed on a digitising pad connected to an Apple II microcomputer. In-house purpose-written software records the radial position (r) of the sedimenting peak. From a plot ln(r) against time the apparent sedimentation coefficient is obtained. After standardisation, the reduced data were also plotted on Fig. 8. Given that there are only three data points it is hard to draw many conclusions from these results. Certainly the extrapolation to infinite dilution ($s^0_{20,w} = 4.56$ S) yields a sedimentation coefficient similar to that obtained in native buffer. But the strikingly different concentration dependence of the data must surely be due to a combination of at least a degree of disruption in the intermolecular association together with an increased non-ideality as a result of the significant KSCN concentration.

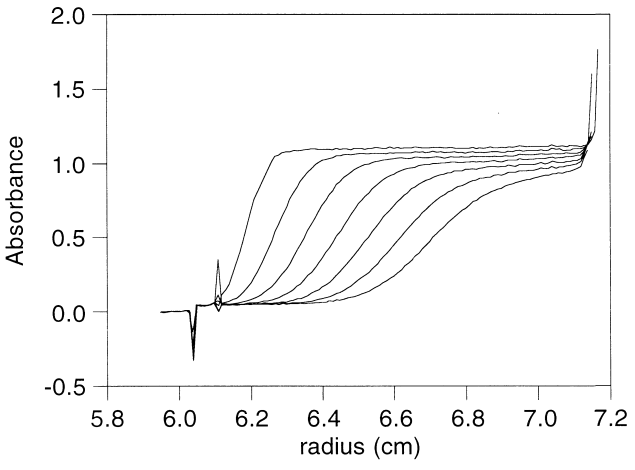

Fig. 7 Sedimentation velocity traces recorded with scanning absorbance optics for DT diaphorase in 10 mM sodium phosphate, pH 7.0 at a loading concentration of 2.95 mg/ml. The rotor speed was 40,000 rpm and the temperature 20 °C

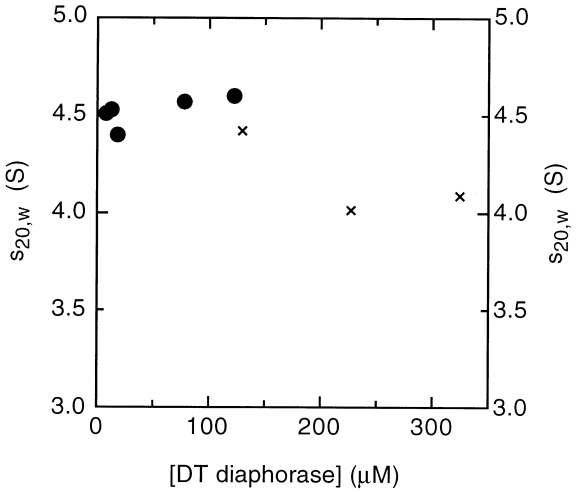

Fig. 8 Weight-average apparent sedimentation coefficient as a function of loading concentration for DT diaphorase in (●) 10 mM sodium phosphate, pH 7.0 buffer; (○) with reduced thiols

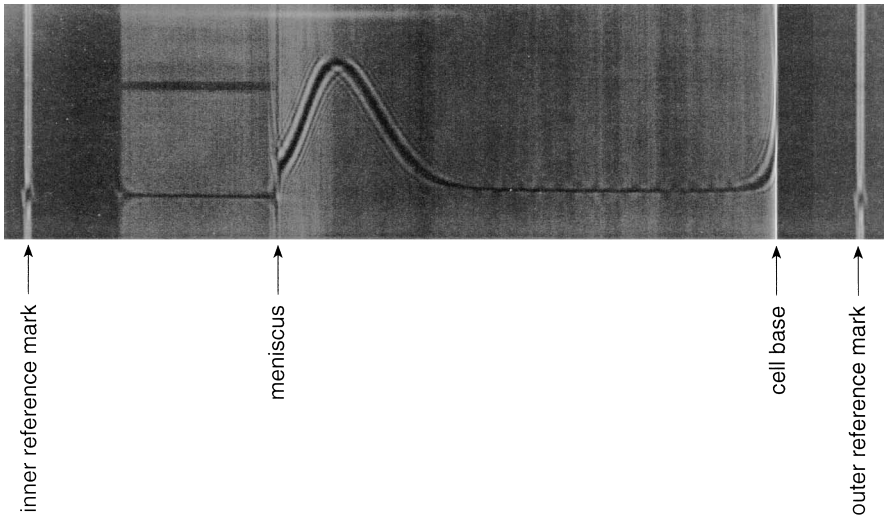

Fig. 9 A typical sedimentation velocity profile for DT diaphorase in 10 mM sodium phosphate, 200 mM KSCN, pH 7.0 recorded with schlieren optics. The rotor speed was 35,140 rpm and the temperature 20 °C. Loading concentration was 10.8 mg/ml

Discussion

The tight self-association of the monomers is consistent with the published crystal structure of DT diaphorase complexes with $NADP^+$ (Li et al. 1995). This reveals that the binding site for the $NADP^+$ cofactor involves residues of the same subunit that binds FAD as well as residues from the other subunit of the dimer (Li et al. 1995). It would be, therefore, mechanistically impossible for DT diaphorase to be active as a monomer. It is as yet uncertain as to whether DT diaphorase would be able to retain its folded conformation as a monomer. Dissociation requires extreme conditions under which the enzyme has been shown to be irreversibly inactivated and destabilised. It may well be consistent with the two-state model proposed by Bowie and Sauer (1989) which states that the native monomer is thermodynamically unstable and does not exist at significant concentrations in equilibrium since quaternary interactions are required for the existence of the folded monomeric state.

There has been no previous observation reported of the existence of a tetrameric form of DT diaphorase. It is interesting to speculate that the dimer-tetramer equilibrium may be significant in some way in a regulatory mechanism of the enzyme. For this reason it is important to know whether the DT diaphorase is indeed active as a tetramer. To investigate the relationship between quaternary structure and activity a concentration-dependent enzyme centrifugation study could be carried out.

Given the assumption made in interpreting the sedimentation equilibrium data it is surprising that the apparent Kd values in Fig. 5 do not decrease with increasing concentration. If the system was in reality a monomer-dimer-tetramer equilibrium, at low concentrations the solute distribution should reveal measurable monomer with a concomitant decrease in weight-average mass and an increase in Kd. A further test of this proposed model will be possible when the atomic coordinates of DT diaphorase become available (the structure has been solved by Li et al. 1995). Then the sedimentation coefficient can be accurately predicted for the DT diaphorase monomer via hydrodynamic bead modelling strategies (see Byron 1997; Spotorno et al. 1997). The dimer can also be modelled in order to generate the true infinite dilution sedimentation coefficient in Fig. 8. Similarly values can be simulated for various tetramer models. These sedimentation coefficients can then be used to fit the data of Fig. 8 to a dimer-tetramer equilibrium following the approach of Gilbert and Gilbert (1973) which has been extended by Rowe (1977, 1992) and is implemented in locally written software (SA-Plot, P. Fellows, University of Leicester, 1995) so that a series of dissociation curves of different Kd can be fitted to the reduced experimental data (see Silkowski et al. 1997). It will be interesting to compare the Kd estimated from this approach with that measured here. Alternatively, conformations for the tetramer can be proposed on the basis of Gilbert and Gilbert-type modelling if Kd = 247 (±16) μM is used.

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