SUBUNIT STRUCTURE AND PHYSICAL PROPERTIES OF AN NADP (H) -BINDING PROTEIN FROM HUMAN ERYTHROCYTES

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1. Introduction

We have reported the occurrence in human erythrocytes of a specific NADP (H) -binding protein, designated FX [1], which was recently purified by affinity chromatography and obtained in a homogeneous form [2]. Despite its unusually high affinity toward both NADP and NADPH (the dissociation constants being K_{NADP} 1.8×10^{-7} M and K_{NADPH} 1×10^{-8} M), purified FX is not associated with any known NADP (H)-dependent dehydrogenase activity nor with a number of enzyme activities screened for this purpose [2]. Since these negative findings could result from inactivation during the procedure of purification, we were prompted to investigate the physical properties of purified FX in order to obtain a reliable comparison with other enzyme proteins that are metabolically related to the NADP/NADPH system. Another reason for undertaking such molecular studies was the attempt to clarify previous uncertainties on the subunit structure of FX in solution which affected seriously the most typical property of this protein, namely combination with NADP (H) : thus, the actual stoichiometry of NADP (H) binding (i.e., not simply extrapolated to the single polypeptide chain) was still open to question.

Several lines of evidence, which are described in this report, indicate that native FX is a dimer of 68 000 M_r. This and other properties, besides providing conclusive evidence for the intrinsic half-site reactivity of FX toward NADP (H), are consistent with substantial differences with known NADP (H)-binding proteins present in human erythrocytes.

2. Materials and methods

FX was purified as described elsewhere [2]. Protein was evaluated according to Lowry et al. [3], or by ultraviolet absorbance, or by the alkaline hydrolysis procedure [4]. Homogeneity of FX preparations was routinely checked as reported previously [2].

The content of sialic acid was evaluated according to Svennerholm [5]. Hexosamines were determined by the procedure of Moore and Stein [6]: the hydrolysis reaction for separation of hexosamines was carried out under nitrogen in 4 N HCl for 7 h at 105°C. Trimethylsilyl derivatives of neutral sugars were analyzed as reported by Cetta et al. [7].

All ultracentrifugation studies were performed in a Beckman Spinco model E centrifuge equipped with an RTIC unit. Sedimentation velocity was carried out using schlieren optics, while high speed sedimentation equilibrium runs were performed according to Yphantis [8] with the use of interference optics. The buffer used throughout all experiments was 0.05 M Na phosphate, pH 7.0, containing 0.1 mM EDTA and 0.01 mM NADP (Buffer A), unless otherwise specified. The buoyant density of FX was determined by equilibrium density-gradient ultracentrifugation [9], using a 12 mm Kel-K cell: centrifugation was at 25°C for 18 h at 44 000 rev/min using a protein concentration of 2.5 mg/ml (Buffer A). Banding of the protein was evaluated by ultraviolet absorption.

Sodium dodecylsulfate slab electrophoresis on polyacrylamide gels in the presence of β-mercaptoethanol was carried out as described previously [2]. The NADP content of native FX preparations was estimated accord-

ing to the procedure reported elsewhere [2].

The Stokes radius of FX was evaluated by gel chromatography on Sephadex G-100, using Buffer A as eluant and the following standard proteins: human erythrocyte glucose 6-P dehydrogenase, purified in our laboratory [2], bovine serum albumin (Sigma), yeast, horse liver and beef liver alcohol dehydrogenase (Boehringer), bovine pancreas ribonuclease (Sigma) [10].

Native FX, dissolved in Buffer A containing either NADP or NADPH, was air dried in 1% Na silicotungstate negative stain on thin carbon films, and electron micrographs were prepared at a nominal magnification of X 100 000 in a JEM 100 C electron microscope. Magnification was accurately calibrated with catalase crystals and measurements taken on enlarged prints.

3. Results

3.1. *Absorption spectrum and carbohydrate analysis of FX*

The absorption spectrum of purified FX showed a peak at 274 nm and a slight shoulder at 292 nm, while no absorption was detectable in the visible region. The molar extinction coefficient of the homogeneous protein, estimated by coupling the spectral analyses with the results of protein determinations and of molecular weight measurements (see below), was $\epsilon_{M,274}$ 82 280 ($A^{1\%}_{274}$ 12.1).

No detectable content of carbohydrate (sialic acids, hexosamines and neutral sugars) could be observed in all preparations insofar examined of homogeneous FX.

3.2. *Physical properties*

Figure 1 shows the result of a typical high speed sedimentation equilibrium experiment performed with an electrophoretically homogeneous preparation of native FX. The molecular weight, calculated on the basis of a partial specific volume of 0.724 ml/g (as determined by amino acid analysis [11]), was 68 900. Since this FX preparation contained 0.5 mol NADP/34 000 M_r, (i.e., the molecular weight estimated under dissociating conditions), the resulting stoichiometry is 1 mol NADP/dimeric FX.

Sedimentation velocity experiments, carried out in a wide range of solvent conditions (including replacement of NADP by NADPH, omission of EDTA, raising

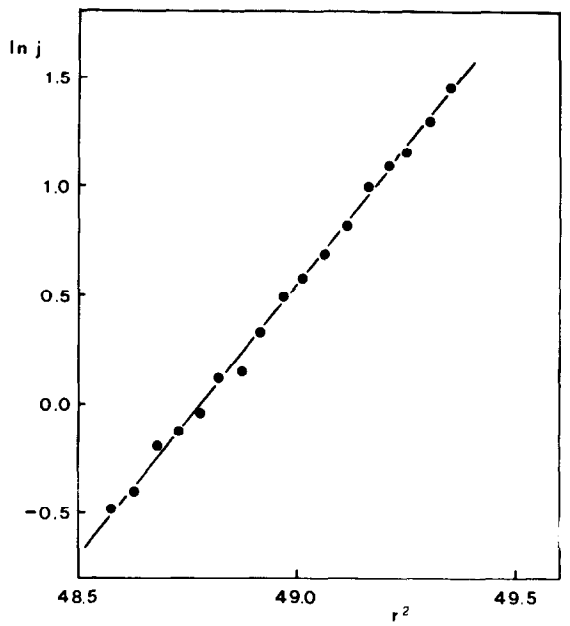

Fig.1. High speed sedimentation equilibrium of FX [8]. The protein (0.5 mg/ml of Buffer A) was subjected to centrifugation at 24 000 rev/min for 12 h at 20°C. Attainment of the equilibrium was checked after 3 additional h at the same speed. The actual content of NADP, estimated by the spectrofluorometric procedure described previously [2], was 0.48 mol/33 000 mol. wt.

Table 1
Molecular parameters of FX

$A^{1\%}_{274}$	12.1
$s_{20,w}$ (10^{-13} s)	4.67 ± 0.15
Stokes radius (10^{-8} cm)[a]	32.3 ± 1.1
$D_{20,w}$ (10^{-7} cm²/s)[b]	6.2 ± 0.3
Molecular weight[c]	68 900 ± 1000
Molecular weight SDS–protein	33 700 ± 1000
Partial specific volume (ml/g)[d]	0.724
f/f_o[e]	1.19
Buoyant density (g/ml)	1.296
Physical dimensions[f]	41 × 82 Å ± 5%

[a] Determined by gel chromatography on Sephadex G-100 according to Ackers [15]
[b] Calculated from spreading of the sedimenting boundary [13], at an initial protein concentration of 2 mg/ml in Buffer A
[c] Determined by high speed sedimentation equilibrium in Buffer A (protein concentration, 0.5 mg/ml)
[d] Determined by amino acid analysis [11]
[e] Calculated according to Siegel and Monty [16]
[f] Measured on enlarged electron micrographs like fig.2

the pH up to 8.6 or lowering it down to 5.8, presence of 1% Na silicotungstate (as in electron microscopy)), yielded an average value of the sedimentation coefficient, $s_{20,w}$, of 4.67 S.

The diffusion coefficient, determined in the ultracentrifuge by Fujita's method [12] modified by Van Holde [13], was $D_{20,w}$ 6.2×10^{-7} $cm^2 \cdot s^{-1}$. The Stokes radius of FX, obtained by gel chromatography (see Materials and methods), was 32.2 Å. Using this value in the Stokes-Einstein equation [14], the diffusion coefficient ($D_{20,w}$) was calculated to be 6.6×10^{-7} $cm^2 \cdot s^{-1}$.

Combining the actual values of both the sedimentation and diffusion coefficients in the Svedberg equation and taking a partial specific volume of 0.724 ml/g [11], a molecular weight of 68 000 ± 2000 was calculated, which compares very satisfactorily with the figure obtained by sedimentation equilibrium. The frictional ratio was estimated to be 1.19, this indicating that FX in solution behaves as a globular protein.

Table 1 summarizes the physical and hydrodynamic parameters of human FX.

3.3. *Electron microscopy*

Measurements of the remarkably homogeneous molecules seen in fig.2 showed native FX to be elongated, 41 Å wide and 82 Å long with standard deviation of 5%. Most of the molecules appeared narrower in the middle, and together with the axial ratio of exactly 2:1 this dumbell shape suggests two near-spherical subunits each of 41 Å diameter. If this assumption of shape is correct, then each sphere

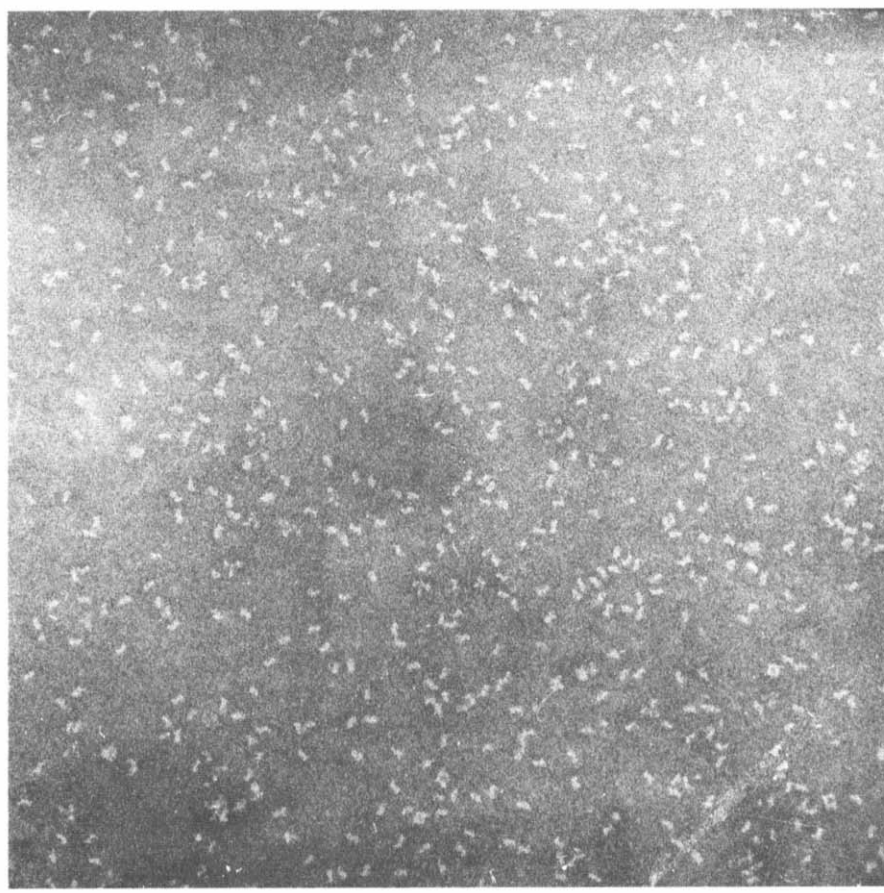

Fig.2. Electron micrograph showing a typical spread of native FX molecules stained with 1% aqueous sodium silicotungstate (× 200 000).

would have a molecular weight of 30 000 ± 15%, using the above value of partial specific volume. Other less plausible assumptions of shape (e.g., two touching cubes) would not significantly alter this value.

4. Discussion

The molecular weight of the single polypeptide chain of FX is 33 000 ± 1000. The results obtained by ultracentrifugation and electron microscopy clearly indicate that native FX is a dimeric protein, while ruling out other aggregation forms over a wide range of experimental conditions. Therefore, the stoichiometry of combination with NADH (H) is one mol dinucleotide/FX molecule, a result indicating that 'half-site reactivity' is an inherent molecular property of FX. Alternative explanations, such as the co-purification of two distinctive populations of monomeric molecules displaying 'all' versus 'none' reactivity with NADP (H), are in fact excluded by the present findings.

Inspection of the physical parameters of FX shows unequivocal differences with those of the major NADP-binding proteins known to be present in human erythrocytes, i.e., glucose 6-phosphate dehydrogenase [10,17], gluconate-6-phosphate dehydrogenase [18], glutathione reductase [19] and methemoglobin reductase [20,21]. These differences strengthen the view that FX is a hitherto unknown protein of the erythrocyte. Accordingly, the present data can be considered as a structural pre-requisite in order to approach the biological function of this holoprotein.

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