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Quaternary structure of human nucleoside diphosphate kinase isoforms HA and HB in solution

Sabine Schaertl*

Max-Planck Institut für Biophysikalische Chemie, Abteilung für Molekulare Genetik, Postfach 2841, 37018 Göttingen, Germany

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Abstract Human isoforms of nucleoside diphosphate kinase, NDPK-HA and NDPK-HB, have been expressed in *E. coli* and purified. Their apparent molecular masses have been determined by FPLC gel filtration. Absolute molecular masses were measured by equilibrium ultracentrifugation and sedimentation coefficients determined from the sedimentation velocity. Under near-physiological conditions, NDPK-HA has a mass of 101 ± 3 kDa, close to that calculated for a hexamer (102.11 kDa), whilst NDPK-HB has a mass of 71 ± 3 kDa, close to a tetramer (68.67 kDa). The sedimentation coefficients, 5.15 ± 0.2 and 3.41 ± 0.1 × 10^{-13} s, for HA and HB also indicate a hexamer and a tetramer respectively. This suggests, although the crystal structure shows a hexameric quaternary arrangement [Webb et al. (1995) J. Mol. Biol. 251, 574–587], that NDPK-HB forms tetramers in solution like bacterial NDPK [Williams et al. (1993) J. Mol. Biol. 234, 1230–1247].

Key words: Nucleoside diphosphate kinase; Quaternary structure; Analytical ultracentrifugation; Gel filtration

1. Introduction

Nucleoside diphosphate kinases (NDPK) catalyze the transfer of the γ-phosphate between nucleoside triphosphates and nucleoside diphosphates. There is a single nucleotide binding site and catalysis involves a phosphorylated intermediate of the protein in a ping-pong mechanism [1,2]. NDP kinases were purified from various sources some time ago and many of their biochemical and kinetic properties characterized [2]. The protein has recently come back into focus again for its postulated role in tumor suppression [3,4]. There are predominantly two human isoforms, known as NDPK-HA [5] and NDPK-HB [6], which share 88% amino acid sequence identity with a monomer mass of 17 kDa [7] but differ in their isoelectric points with 6.13 for NDPK-HA and 8.64 for NDPK-HB. These two isoforms form mixed complexes in vivo [7,8] which has led to the isolation of six NDP kinase oligomers from human erythrocytes, separable by isoelectric focusing, with molecular masses, determined by gel filtration, between 80 and 100 kDa [9,10]. They also have differing catalytic properties such as the Michaelis-Menten K_ms for ATP, ADP, GTP or GDP and the linearity of Arrhenius plots of reaction velocity [9,10]. Monomeric human NDP kinase has less than 5% of the enzymatic activity of single-isoform oligomers (S. Schaertl, unpublished results) emphasizing the importance of the complexed form. The human NDP kinase isoform HB has been reported to bind to specific regions in the human c-*myc* promoter [11] and preferentially to single-stranded poly-pyrimidine tracts [12], a function for which NDP kinase activity is not required [13].

By molecular sieving, prokaryotic NDP kinase from *Escherichia coli* has been found to be a tetramer [14] and eukaryotic NDP kinases, including the two human isoforms NDPK-HA and -HB, have been determined to be hexamers [7,8,15,16]. 30 years ago Yue et al. [17] carried out detailed studies on NDPK from *Saccharomyces cerevisiae* using sedimentation techniques on the Spinco Model E analytical ultracentrifuge. They measured a molecular mass of 102 ± 2 kDa, close to the mass of a hexamer which is 103.8 kDa for *Saccharomyces cerevisiae*. Palmieri et al. [16] continued these studies, varying the pH from 5.6 to 7.9, again showing hexameric assembly of yeast NDP kinase. Williams et al. [18] crystallized NDPK from *Myxococcus xanthus* and measured its mass in solution by equilibrium ultracentrifugation under similar conditions to those used for growing the crystals. They determined a mass of 66 kDa; this compares to 64 kDa calculated for the tetramer and found in the crystals.

The currently known high-resolution crystal structures of NDPK are: tetrameric: *Myxococcus xanthus* [18]; hexameric: *Dictyostelium discoideum* [19], *Drosophila melanogaster* [20] and *Homo sapiens* nm23-H2, NDPK-HB [21]. However, Hamby et al. [22] report NDPK-HB to be tetrameric in solution and preliminary gel filtration studies in our laboratory also suggested a smaller complex than a hexamer for NDPK-HB. These controversies have prompted us to investigate the two human isoforms NDPK-HA and -HB, using recombinant proteins, to clarify their structures in solution under near-physiological conditions. We expressed and purified the proteins to >90% homogeneity and determined their molecular masses by electrospray mass spectroscopy (EMS). Their masses in solution under near-physiological conditions were measured by FPLC molecular sieving and analytical ultracentrifugation.

2. Materials and methods

2.1. Chemicals and proteins

Chemicals were purchased from Boehringer, Mannheim, or from Sigma. The genes nm23-H1 and nm23-H2 encoding NDPK-HA and -HB (from M.-L. Lacombe, Paris) were subcloned into the vector pJC20 [23] and sequenced. This vector was used to transform expression bacteria: strain BL21 DE3 *E.coli*. 2–5 l of bacterial culture were induced at an optical density (OD) at 600 nm of 0.6 with 1 mM IPTG for 5 h at 37°C, harvested by centrifugation at 6000 rpm and sonicated. Purification was performed using modified protocols [24,25] with fresh DTT added every day. The lysate was centrifuged for 45 min with a Sorvall RC-5B centrifuge in a SS-34 rotor at 10000 rpm at 4°C and the supernatant was dialysed overnight against 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DDT, and then loaded on a Q-Sepharose ion exchange column (Pharmacia). NDPK-HB remained in

*Corresponding author. Fax: (49) (551) 201 1718.
E-mail: SSCHAER@uli.dnet.gwdg.de

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the flow-through but NDPK-HA and *E. coli* NDP kinase bound. NDPK-HA was eluted with a gradient of 0–0.5 M NaCl and further purified by FPLC with a blue Sepharose column (CL 4B, Pharmacia) to which it bound and was eluted with a gradient of 0–2 M NaCl. The protein solutions were analyzed by electrophoresis on 15% SDS-PAGE gels. These showed that both NDPK-HA and -HB were more than 90% pure. They were stored at 4°C as a precipitate in 90% ammonium sulfate or at −20°C in 50% glycerol/near-physiological buffer. Proteins were sedimented by centrifugation (Eppendorf 5415, 15 min at 14000 rpm), dissolved and desalted in a PD10 gel filtration column (Pharmacia) in the experimental buffer. Two buffers were used: TB which was 50 mM Tris-HCl pH 8.0 (20°C), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT; and PB for near-physiological conditions, 50 mM MOPS pH 7.2, 100 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT.

2.2. Analytical procedures

Protein concentration was estimated using calculated extinction coefficients ε of 1.315 cm^2 mg^{-1} for NPDK-HA and 1.299 cm^2 mg^{-1} for NDPK-HB at 280 nm [26]. NDP kinase activity was measured in a standard coupled enzyme assay [15,27] with 5 mM ATP and 0.5 mM TDP as substrates. The product ADP together with 2 mM PEP was converted by pyruvate kinase to ATP and pyruvate. Pyruvate and 0.2 mM NADH were turned over by lactate dehydrogenase to lactate and NAD^+. The reaction was followed by optical absorption of NADH at 340 nm. Activity of 1 unit is defined as the turnover of 1 μmol substrate in 1 min per mg of protein. Molecular sieving was performed in a Pharmacia LKB Fast Performance Liquid Chromatography system with a Superose 6 HR 10/30 column at a flow rate of 0.5 ml min^{-1}; protein was detected by absorbance at 280 nm. A gel filtration standard marker set from BioRad with bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), vitamin B-12 (1.35 kDa) was used for calibration. The absolute molecular masses of the proteins were determined by equilibrium centrifugation in a Beckman XL-A analytical ultracentrifuge using an An 60 Ti rotor with three sample cells, each containing 110 μl of sample and 125 μl of buffer for reference. NDPK-HA and -HB were compared in adjacent cells. They were equilibrated first at 9000 and then at 12000 rpm. Radial scans were performed at 280 nm with a step size of 0.001 cm and data were processed with Origin (Microcal) software on a PC. Sedimentation coefficients were also determined from the sedimentation velocity of the same samples measured at 40000 rpm.

Molecular masses of NDPK-HA and NDPK-HB were measured by EMS on samples of 50 pmol. A Micromass Platform spectrometer was used as described by Aitken et al. [28].

3. Results and discussion

The molecular masses calculated from the amino acid sequence (using GCG software) were 17017.58 Da for NDPK-HA and 17166.89 Da for NDPK-HB. EMS was used to check the absolute molecular masses of the two recombinant proteins. They were each found to be a single protein population with molecular mass within 1 Da of that calculated (Table 1). This showed that the N-terminal methionine of both proteins was post-translationally removed by the *E. coli* expression system. SDS-PAGE gels (not shown) demonstrated that NDPK-HA migrated at 20 kDa and NDPK-HB at 19 kDa showing a behavior that is known for the two human NDP kinase isoforms [7]. Both isoforms had a specific activity (turnover rate per active site) of 600–700 U mg^{-1} (170–200 s^{-1}). The calculated molecular masses for hexamers of NDPK-HA and -HB were 102.11 and 103.00 kDa respectively, and for tetrameric HA and HB, 68.07 and 68.67 kDa.

For analysis by molecular sieving the proteins were passed through a gel filtration column under different conditions always together with the standard markers. NDPK-HA and -HB eluted in a major peak at different elution volumes and were fitted to different masses as listed in Table 2. A minor peak fitting to the mass of a monomer could be seen under some conditions, mainly with NDPK-HB. Under near-physiological conditions the proteins HA and HB migrated at 84–86 kDa (HA) and 54–58 kDa (HB), independent of the amount of MOPS (25–50 mM, pH 7.2–7.3), DTT (0–15 mM) or nucleotide (0.4 mM ADP) present, showing consistently ~16% (HA) or ~19% (HB) less mass than expected for the hexameric HA or the tetrameric HB. Incubation with 1 mg/ml ovalbumin did not change this result. However, in buffer TB the proteins migrated at 103 kDa (HA) and 50 kDa (HB). In a buffer which simulated the conditions used for crystallization [21], 50 mM Tris-HCl pH 8.5, 0.5 M NaCl, 20 mM DTT, both proteins showed a different behavior with respect to the marker proteins migrating at 73 kDa (HA) and 64 kDa (HB). The marker proteins migrated at consistent elution volumes under all conditions.

We determined the absolute molecular masses of the proteins by analytical equilibrium ultracentrifugation. NDPK-HA and -HB were dialysed against TB or PB for at least 12 h. The concentration of the proteins was adjusted to an OD_{280} of 0.5–0.7 (in TB) and 0.8–1.0 (in PB). They were centrifuged at 15°C first at 9000 and then 12000 rpm for 20 h at each

Table 1
Molecular masses of NDPK-HA and NDPK-HB, calculated (using GCG software), measured by EMS and determined by analytical equilibrium ultracentrifugation, and their sedimentation coefficients

Methods	Conditions	Results NDPK-HA	Results NDPK-HB
Calculated molecular mass [kDa]	for monomer	17.0176	17.1669
	for tetramer	68.07	68.67
	for hexamer	102.11	103.00
EMS [Da]	in PB	17018.6 ±1.72	17167.7 ±0.89
Equilibrium ultracentrifugation [kDa]	in TB		
	at 9000 rpm	128.2 ±2.0	71.7 ±0.9
	at 12000 rpm	123.5 ±2.2	67.9 ±0.8
	mean	**126 ±4**	**70 ±4**
Equilibrium ultracentrifugation [kDa]	in PB		
	at 9000 rpm	100.8 ±1.2	70.7 ±1.9, 71.7[a] ±1.3
	at 12000 rpm	100.3 ±0.8	71.4 ±1.0, 71.4[a] ±1.1
	mean	**101 ±3**	**71 ±3**
Sedimentation coefficient [10^{-13} s]	in TB	5.1 ±0.2	3.2 ±0.1
	in PB	5.15 ±0.2	3.41 ±0.1

Errors quoted are the random errors from the fitting procedure, except the means which include the systematic errors in calculating specific volumes and buffer densities.
[a]Second sample of HB without DTT present.

Table 2
Apparent molecular masses of NDPK-HA and NDPK-HB determined by FPLC gel filtration (Superose 6)

Conditions	NDPK-HA [kDa]	NDPK-HB [kDa]
25 mM MOPS pH 7.3, 100 mM KCl, 1 mM EDTA, 2 mM $MgCl_2$ 0/1/15 mM DTT		
1 mM DTT, 0.4 mM ADP	85–86 ± 9	54–58 ± 6
50 mM Tris-HCl pH 8.5, 0.5 M NaCl, 20 mM DTT	74 ± 7	64 ± 6
TB	103 ± 10	50 ± 5
PB	84 ± 8	58 ± 6

speed until equilibrium was attained. The baselines which were used for fitting the molecular masses were determined after further high speed centrifugation at 40 000 rpm. The data were analyzed using a model of an ideal single species [29] having a molecular mass that gave the best fit (lowest chi-square). This was used throughout. Specific volumes were calculated according to the method in [29]; at 15°C these were 0.7364 and 0.7401 $cm^3\ g^{-1}$ for HA and HB, respectively. Buffer densities at 15°C were 1.00093 for TB and 1.0077 $g^1\ cm^{-3}$ for PB. The fitted molecular masses are shown in Table 1. In experimental buffer TB, HB data were fitted to molecular masses of 71.7 and 67.9 kDa, HA to 128.2 and 123.5 kDa. Under near-physiological conditions in PB, two samples of HB were analyzed, the second having no DTT present. HB fitted to masses of 70.7 and 71.7 kDa at 9000 rpm and 71.4 and 71.4 kDa at 12 000 rpm; HA to 100.8 kDa at 9000 rpm and 100.3 kDa at 12 000 rpm. The absence of DTT did not appear to change the mass of HB.

In buffer TB the fitted masses of both proteins varied slightly at different angular velocities suggesting the presence of another species [30]. HA showed a high degree of aggregation which is consistent with the gel filtration experiments where HA could be fitted to a much larger mass than determined under all other conditions. The variability of these results may be due, at least in part, to electrostatic interactions at low ionic strength (~35 mM) of buffer TB [30]. Under near-physiological conditions the masses fitted for HA and HB were very close to those calculated for the hexameric HA and tetrameric HB (Table 1), the small differences could be due to varying baselines because of oxidation of DTT (which then absorbs at 280 nm), small amounts of contaminating proteins or aggregation.

The optical density profiles at both angular velocities were then fitted to the calculated masses of both hexamer and tetramer of HA and HB (Fig. 1). This shows clearly that reasonable fits (low chi-square) are obtained only for hexameric HA and tetrameric HB.

Under near-physiological conditions sedimentation coefficients ($\times 10^{-13}$ s) of 5.15 for HA and 3.41 for HB were measured (Table 1). If we assume that both proteins have near-spherical geometry, the ratio of their sedimentation coefficients should be proportional to their masses [30]. This ratio is very close to 1.5:1 as required for hexamer:tetramer. The sedimentation coefficients obtained under non-physiological conditions (in TB) were 5.1 for HA and 3.2 for HB, apparently not showing aggregation of HA. Sedimentation coefficients measured for yeast NDP kinase [16] were between 5.58 and 5.82 at pH 5.6–7.9 indicating a less tightly packed structure than that of NDPK-HA.

All experiments under near physiological conditions were repeated with recombinant protein from the same batches as were analyzed by EMS. As no significant differences were seen between these results and those using previous batches all results have been pooled together in Tables 1 and 2.

The apparent molecular masses that have been determined in several approaches are consistent under near-physiological conditions fitting to a hexameric assembly of NDPK-HA and a tetrameric assembly of NDPK-HB. Under non-physiological conditions both proteins give inconsistent molecular masses indicating that conditions such as pH or ionic strength could affect the quaternary structure. As NDPK-HB is able to form hexamers when associating with NDPK-HA [8,10] it is plausible that the protein may be pushed into the hexameric form under conditions of high salt at pH 8.5, whereas it clearly exists as a tetramer in the pure HB isoform under near-physiological conditions.

The results from analytical ultracentrifugation show conclusively that the tetrameric mass of NDPK-HB is not due to a mixture of monomers and hexamers. Such a mixture would have yielded two distinct sedimentation coefficients corresponding to the monomer and hexamer. Also the forms of the protein concentration profiles against radius measured during equilibrium ultracentrifugation at differing angular velocities did not permit fitting to a mixture of monomers and hexamers but only to a single tetrameric molecular species.

A more general and complicated question concerns the nature of the mixed forms between NDPK-HA and -HB, the quaternary structure might be dependent on the presence of HA and only one subunit of HA may be sufficient to turn NDPK-HB into a hexamer. It is also possible that the ability to bind DNA [11] is restricted to the tetrameric NDPK-HB.

The gel filtration results and sedimentation coefficient of NDPK-HB indicate a tightly packed spherical quaternary structure and the equilibrium centrifugation a tetrameric mass. NDPK-HA, however, appears to be hexameric under identical experimental conditions. We therefore suggest that NDPK-HB, under physiological conditions, rather than being in the hexameric form seen in the crystal structure [21], exists with a similar structure to that seen in crystals of tetrameric NDP kinase of *Myxococcus xanthus* [18] where two dimers are arranged cross-like in a tightly packed oligomer.

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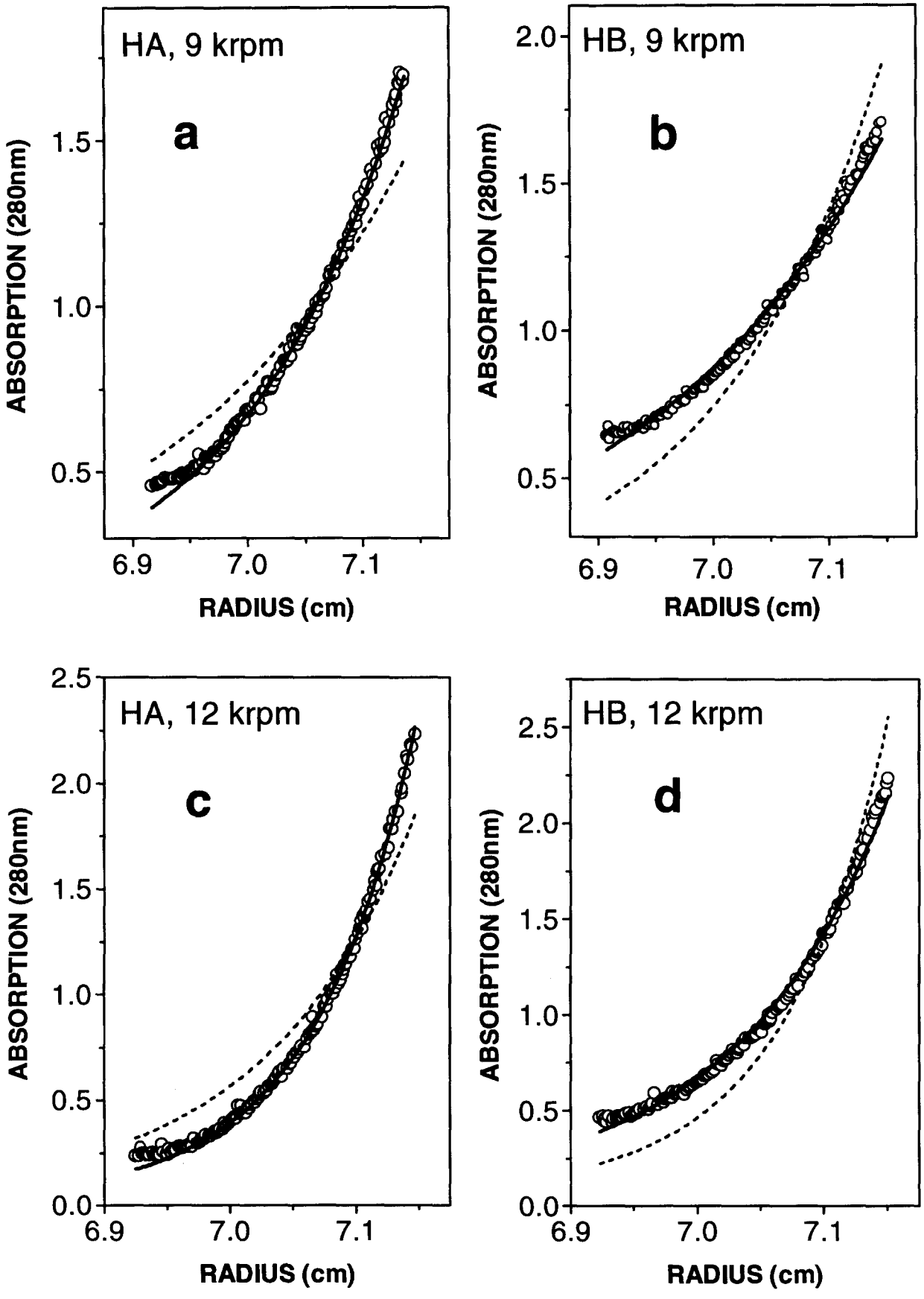
Fig. 1. Equilibrium ultracentrifugation under near-physiological conditions. Circles are the optical density distribution at 280 nm as a function of the sample cell radius at equilibrium for NDPK-HA (a) and NDPK-HB (b) at 9000 rpm and NDPK-HA (c) and NDPK-HB (d) at 12 000 RPM. HA (a,c) was fitted to the calculated mass of the hexamer and HB (b,d) to that of the tetramer as shown in each case by the solid line through the data. The broken lines are best fits to the calculated mass of a tetramer to HA (a,c) and to that of a hexamer of HB (b,d). Buffer conditions were: 50 mM MOPS pH 7.2, 100 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT at 15°C.


HA, 9 krpm
a
HB, 9 krpm
b
HA, 12 krpm
c
HB, 12 krpm
d
ABSORPTION (280nm)
RADIUS (cm)
0.0
0.5
1.0
1.5
2.0
2.5
6.9
7.0
7.1

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