Inner and Outer Arm Axonemal Dyneins from the Antarctic Rockcod *Notothenia coriiceps*[†]

Stephen M. King,[‡,§] Silvio P. Marchese-Ragona,[‖,⊥] Sandra K. Parker,[‡] and H. William Detrich, III*[,‡]

Department of Biology, Northeastern University, Boston, Massachusetts 02115, and Department of Molecular and Cell Biology, Pennsylvania State University, University Park, Pennsylvania 16802

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ABSTRACT: Adaptive compensation of enzymatic activities is common among cold-living poikilotherms. Their enzymes often demonstrate higher activities at low temperatures than do homologs from temperate or thermophilic species. To understand the molecular features necessary for cold adaptation of microtubule motor proteins, we have initiated studies of the flagellar dynein ATPases of Antarctic fishes (body temperature range = −1.8 to +2 °C). Dyneins were isolated by high-salt extraction of demembranated sperm axonemes from the Antarctic yellowbelly rockcod, *Notothenia coriiceps*. Although solubilization of inner arms was incomplete, an inner arm dynein was recognized as a discrete complex containing one major dynein heavy chain (DHC) and sedimenting through sucrose gradients at ∼12 S. Like inner arm dyneins from *Chlamydomonas*, the fish complex contained an actin-immunoreactive protein of 43 kDa and a 30-kDa protein. One isoform of the inner arm DHC gene family of *N. coriiceps* was detected by the polymerase chain reaction, and Southern analysis established that this DHC gene is present at one copy per haploid genome. Outer arm dynein was extracted quantitatively by high-salt treatment, contained two DHCs (one major, one minor), and sedimented through sucrose gradients as a polydisperse, aggregating system. Associated with the outer arm DHCs were five presumptive intermediate chains (ICs) of 66−91 kDa, immunologically defined by their cross-reactivity to four monoclonal antibodies specific for ICs from other organisms. The basal (non-microtubule-stimulated) specific ATPase activities of the *N. coriiceps* inner and outer arm dyneins were ∼0.07 and ∼0.04 μmol of P_i min^{-1} mg^{-1}, respectively, at 0 °C, attained their maxima (∼0.1 μmol of P_i min^{-1} mg^{-1}) at 9 and 19 °C, respectively, and at higher temperatures declined substantially. Furthermore, the activities of the fish dyneins at temperatures ≤15 °C were significantly larger than that of outer arm dynein from the mesophile *Tetrahymena*. These results suggest that the greater catalytic efficiencies of *N. coriiceps* inner and outer arm dyneins at low temperatures are due to enhanced polypeptide flexibility in the active sites of their protein subunits. We conclude that temperature adaptation of flagellar dyneins from Antarctic fishes is compatible with substantial conservation of primary and quaternary structure.

Flagellar dyneins are large, macromolecular ATPases that form the inner and outer arms attached to the outer doublet microtubules of flagellar and ciliary axonemes [reviewed by Witman et al. (1994)]. These "minus-end-directed" motors generate the sliding forces that produce flagellar bending by coupling the hydrolysis of ATP to cyclic interaction of dynein complexes with adjacent doublet microtubules. In mammals, the absence of flagellar dynein arms is associated with severe respiratory disease and with male infertility (Afzelius, 1979; Baccetti et al., 1980; Zamboni, 1987). Clearly, it is important to understand the structure, subunit composition, mechanism, and regulation of vertebrate dyneins, but these mechanoenzyme complexes have proven difficult to purify in quantity from mammals. Thus, our current understanding of these mechanochemical enzymes derives largely from studies of protistan and invertebrate dyneins (Witman et al., 1994).

Dyneins are composed of heavy (∼520 kDa), intermediate (∼50−150 kDa), and light (∼6−25 kDa) chains (Witman et al., 1994). In electron micrographs, outer arm dyneins appear as two or three globular heads (∼12 nm) that are connected by thin filaments to a common base, and they are organized uniformly along the axoneme at 24-nm intervals. The number of heads in an outer arm complex equals its content of dynein heavy chains (DHCs).[1] Thus, the three-headed outer arm dyneins of protistans (∼1.7 × 10^3 kDa) contain three heavy chains, whereas two-headed outer arms from other organisms (∼1.25 × 10^3 kDa) possess two. Although less well characterized, inner arm dyneins appear to be more heterogeneous (Witman et al., 1994), with three distinct classes (I1−I3), two (I2 and I3) of which may differ in polypeptide composition both within the 96-nm inner arm longitudinal repeat and at longer intervals along the axoneme (Piperno et al., 1990; Piperno & Ramanis, 1991; LeDizet & Piperno, 1995).

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* To whom correspondence should be addressed at the Department of Biology, Northeastern University, 414 Mugar Hall, 360 Huntington Ave., Boston, MA 02115. Phone: (617) 373-4495. Fax: (617) 373-3724. E-mail: Iceman@neu.edu.

[‡] Northeastern University.

[§] Present address: Department of Biochemistry, University of Connecticut Health Center, 263 Farmington Ave., L-3049, Farmington, CT 06032.

[‖] Pennsylvania State University.

[⊥] Present address: Chapman Instruments, 175 Research Blvd., Rochester, NY 14623.

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[1] Abbreviations: DHC, dynein heavy chain; IC, intermediate chain; LC, light chain; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

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To develop a model system for study of *vertebrate* flagellar dyneins, Gatti et al. (1989) selected sperm of the trout *Salmo gairdneri*. Trout sperm is abundant, relatively simple in structure, and lacks an acrosomal vesicle and its associated proteases. From this source, they purified and characterized extensively the outer arm dynein complex (Gatti et al., 1989; King et al., 1990). This 19S, two-headed particle contains two heavy chains (α and β), five intermediate chains (IC 1−5, 57−85 kDa), and six light chains (LC 1−6, 6−22 kDa). At room temperature, trout dynein displays a strong preference for ATP as substrate nucleotide, with a specific MgATPase activity of 1.1 ± 0.3 μmol min^{-1} mg^{-1} and an apparent K_M for $MgATP^{2-}$ of 40 ± 16 μM. Interestingly, the inner arm dyneins of trout axonemes resist extraction by conventional methods.

To learn more about vertebrate dyneins, with particular emphasis on the inner arms, we have investigated the sperm dyneins of the Antarctic yellowbelly rockcod, *Notothenia coriiceps*. When gravid (Austral Fall, April−May), *N. coriiceps* males devote as much as 20% of their body mass to the production of sperm: more than 100 mL of semen at greater than 10^{12} sperm/mL can be obtained from a single specimen. Like trout sperm, the structure of the sperm is simple, and axonemes free of accessory structures are readily obtained.

In this report, we describe the structural and functional properties of inner and outer arm dyneins isolated from sperm flagella of *N. coriiceps*. The inner arm dynein complex, the first to be purified to homogeneity from a vertebrate, is similar in subunit composition to the flagellar inner arm dyneins of *Chlamydomonas*. Furthermore, we demonstrate the presence of a single-copy gene for one inner arm DHC isoform. The outer arm complex, like trout outer arm dynein, contains two DHCs and five ICs. The temperature dependence of the basal ATPase activities of the inner and outer arm dyneins suggests that high catalytic efficiency at low temperature has been achieved by molecular adaptations that enhance conformational flexibility at their active sites. We propose that the flagellar dyneins of *N. coriiceps* provide ideal experimental models for analysis (1) of cold adaptation of microtubule motor function and (2) of the functional and regulatory properties of both *inner* and *outer* arms in vertebrates. Preliminary reports of some of this work have appeared (King & Detrich, 1993; Marchese-Ragona & Detrich, 1990, 1991).

EXPERIMENTAL PROCEDURES

Collection of Fish. Specimens of the Antarctic yellowbelly rockcod, *N. coriiceps*, and the blackfin icefish, *Chaenocephalus aceratus*, were collected by bottom trawling from the *R/V Polar Duke* near Low and Brabant Islands in the Palmer Archipelago. They were transported alive to Palmer Station, Antarctica, where they were maintained in seawater aquaria at −1 to +1 °C.

Isolation and Extraction of Sperm Axonemes. The procedures used to isolate sperm axonemes from mature male *N. coriiceps* (1.5−3 kg total body weight) and to extract dynein arms from the axonemes are based on methods previously developed for trout sperm outer arm dynein (Gatti et al., 1989; King et al., 1990). Briefly, a supernatant fraction enriched in sperm tails was obtained by dilution of fresh, diced testis with an equal volume of HMEN [20 mM Hepes−NaOH (pH 7.4), 5 mM $MgSO_4$, 0.5 mM EDTA, and 100 mM NaCl], homogenization of the testis in a Dounce tissue grinder (4 strokes, tight-fitting pestle), and centrifugation (2000*g*, 5 min, 4 °C) of the homogenate to pellet sperm heads. Aliquots (30 mL) of the resulting supernatant were underlayed with 15-mL cushions of 25% (w/v) sucrose in HMEN and centrifuged (2000*g*, 20 min, 4 °C) to pellet nonaxonemal debris. The supernatants, which contained sperm tails and some membrane fragments, were recovered and centrifuged (12000*g*, 10 min, 4 °C) to pellet the sperm tails. After two washes [resuspension in HMEN followed by centrifugation (12000*g*, 10 min, 4 °C)], sperm tails were demembranated twice by resuspension in TX-HMEN [1% (w/v) Triton X-100 in HMEN], and axonemes were collected by centrifugation (12000*g*, 10 min, 4 °C). Axoneme pellets were washed once by resuspension in HMEN and centrifugation as before.

For most experiments, dyneins were extracted by resuspension of axoneme pellets to a final concentration of ∼2−3 mg/mL in HS-HMEN (HMEN containing 0.6 M NaCl and 1 mM PMSF). Following a 15-min incubation on ice, the suspension was centrifuged (40000*g*, 10 min, 4 °C), and the dynein-containing supernatant was recovered for subsequent sucrose density-gradient purification. A second extraction protocol, low ionic strength dialysis of the axonemal fraction (Pfister & Witman, 1984), was also tested. Demembranated axonemes were resuspended in 6 mL of LIS buffer [5 mM Tris·HCl (pH 8.0), 0.5 mM EDTA, 1 mM DTT, and 1 mM PMSF] and dialyzed for 24 h against 4 L of LIS (4 °C).

Sperm axonemes were also prepared from semen that had been collected by stripspawning of live males. Care was exercised to prevent contamination of the milt by seawater or urine. Sperm (10 mL) were diluted in HMEN buffer (40 mL), and sperm tails were detached by Dounce homogenization (see above). Subsequent processing (i.e., removal of heads by centrifugation, extraction of axonemal membranes and dynein complexes) was as described above for whole testis.

Purification of Dyneins by Sucrose Density-Gradient Centrifugation. High-salt extract (3 mL/gradient at 0.5−1.0 mg/mL) was loaded onto 30-mL, 5−25% linear sucrose gradients made in HMEN buffer, and the gradients were centrifuged in a Beckman SW25.1 rotor (90000*g*, 4 °C) for 20−24 h [cf. Gatti et al. (1989) and King et al. (1990)]. Fractions of ∼1 mL were collected from the bottom of each tube. When multiple gradients were processed, equivalent fractions were pooled prior to further analysis. Low-salt extract was centrifuged identically, with the exception that the sucrose gradients were made in LIS buffer.

Ion-Exchange Chromatography. Inner arm dynein, which sedimented at ∼12 S in the sucrose gradients, was purified further either by conventional column chromatography on DEAE-Sephacel (Pharmacia LKB Biotechnology Inc.) or by high-performance liquid chromatography on Mono Q [0.5 × 5 cm column; Pharmacia LKB Biotechnology Inc.; cf. Kagami and Kamiya (1992)]. The sample (1.4 mL at ∼0.4 mg/mL in LIS buffer) was applied to the column, and the column was eluted with a linear gradient of NaCl (0.075−0.75 M) in 0.05 M Tris·HCl (pH 7.6).

Dynein from Tetrahymena. Outer arm dynein (22 S) from cilia of *Tetrahymena* was purified by the method of Porter and Johnson (1983). Aliquots were stored frozen at −70 °C prior to use.

Protein and ATPase Determinations. Protein concentrations were measured by the microprotein assay of Bradford

(1976) with bovine serum albumin as the standard. ATPase activities were assayed by quantitation of the release of inorganic phosphate by the malachite green procedure of Lanzetta et al. (1979).

Electrophoresis and Immunoblotting. High molecular weight proteins were separated by SDS−polyacrylamide gel electrophoresis (Laemmli, 1970) in 4% slab gels containing 4 M urea. Proteins of lower molecular weight were resolved by electrophoresis in 8 and 12.5% SDS−polyacrylamide slab gels. Gels were stained either with Coomassie Brilliant Blue R-250 (Detrich & Overton, 1986) or with silver (Wray et al., 1981).

Electrophoretic transfer of proteins from SDS−polyacrylamide gels to nitrocellulose and immunostaining of the nitrocellulose replicas were performed as described previously (King et al., 1986). Primary, anti-IC monoclonal antibodies are described under Results. Secondary antibodies were alkaline phosphatase conjugates.

Electron Microscopy. Testis tissue, intact sperm, detergent-extracted axonemes, and high-salt-extracted axonemes were fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) buffer for 60 min, postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate (pH 7.4) for 60 min, dehydrated through an acetone series, and embedded in Araldite. Thin sections were stained with methanolic uranyl acetate followed by lead citrate. Samples were examined at 60−80 kV using a Philips EM 300 electron microscope.

PCR Amplification and Southern Analysis of Genomic DNA. A probe for the DHC genes of *N. coriiceps*, NcDHCpcr1, was generated by PCR amplification of genomic DNA using degenerate forward and reverse primers derived from the conserved DHC amino acid sequences GTGKTET (5′-GCGCGAATTCGBACBGGBAAGACB-GAGAC-3′) and CFDEFNR (5′-GCGCCTCGAGCGGT-TRAACTCRTCRAAGCA-3′), respectively (Wilkerson et al., 1994). Following directional subcloning between the *Eco*RI and *Xho*I sites of pBluescript SK+, the DNA product was sequenced on both strands using the dideoxynucleotide chain termination method of Sanger et al. (1977) and T4 DNA polymerase (Sequenase II; United States Biochemical).

High molecular weight nuclear DNA was purified (Sambrook et al., 1989) from the testis tissues of one *N. coriiceps* and one *C. aceratus*. Aliquots (10 μg) of the DNAs were subjected to restriction-endonuclease digestion, the digested DNAs were separated by electrophoresis on horizontal 0.7% agarose slab gels, and DNA fragments were transferred to nylon membranes (MSI) by the method of Southern (1975). The Southern replicas were probed for DHC sequences by hybridization to digoxygenin-labeled NcDHCpcr1 probe (Genius System, Boehringer-Mannheim). Prehybridization and hybridization of the membranes were performed at 65 °C in 5× SSC, 0.1% *N*-lauroylsarcosine, 0.02% SDS, and 1% blocking reagent (Boehringer-Mannheim) for 1 h and 16−20 h, respectively, and the membranes were washed sequentially in buffers of increasing stringency (final wash conditions: 0.5× SSC, 0.1% SDS, 65 °C). Following incubation with anti-digoxygenin, alkaline phosphatase-conjugated Fab fragments, and Lumi-Phos 530 (Boehringer-Mannheim), the chemiluminescent signal of the bound probe/antibody conjugate complex was detected on Kodak XAR-5 X-Omat film.

Chemicals. ATP (equine muscle, <1 ppm vanadium), EDTA, Hepes, PMSF, and SDS were obtained from Sigma. Acrylamide and *N*,*N*′-methylenebis(acrylamide) were products of Bio-Rad Laboratories, and urea (enzyme grade) was purchased from Bethesda Research Laboratories. Taxol (kindly provided by Dr. Matthew Suffness, Natural Products Branch, Division of Cancer Treatment, National Cancer Institute) was prepared as a 10 mM stock in dimethyl sulfoxide and stored at −70 °C. Araldite 502, glutaraldehyde, osmium tetroxide, sodium cacodylate, and uranyl acetate were obtained from Ted Pella, Inc. Tannic acid was supplied by Mallinckrodt. Other chemicals were reagent grade.

RESULTS

Purification of Axonemal Dyneins. (A) Isolation and Extraction of Sperm Axonemes. Spermatozoa, either from minced testis or from ejaculated milt, were deflagellated by brief homogenization. Sperm tails were collected by differential sedimentation, and axonemes were prepared by extraction with 1% Triton X-100. After several washes, dyneins were solubilized by incubation of the axonemes in HMEN containing 0.6 M NaCl. Figure 1 shows electron micrographs of intact sperm, sperm flagella, axonemes, and salt-extracted axonemes from one preparation. Both the inner and outer dynein arms remained associated with the axoneme following membrane removal (Figure 1D). High-salt treatment removed the outer arms almost quantitatively, but extraction of the inner arms was incomplete (Figure 1E). Figure 2 presents an electrophoretic analysis of the axonemal proteins solubilized by the high-salt treatment. On this 8% gel, two DHC bands are discernible in the axonemal fraction (Figure 2, lane AX). [Note that in lower percentage acrylamide gels, four discrete bands can be distinguished in the DHC region (Figure 4, lane AX).] Following high-salt treatment, the upper DHC band was almost completely recovered in the soluble HSE fraction, whereas greater than 50% of the lower band remained associated with the extracted axonemes (lane EA). Together, the morphological and biochemical data suggest that the readily extracted outer dynein arms contain the larger DHC species, whereas the partially extracted inner dynein arms contain the smaller DHC.

The high-salt extract also contained numerous proteins of intermediate and low molecular weight, including tubulin.

(B) Sedimentation Analysis of Extracted Dyneins. Figure 3 shows the fractionation of the high-salt extract by sedimentation through a 5−25% sucrose density gradient. The electrophoretically rapid DHC species (Figure 2, lower DHC band) sedimented as a discrete peak at ∼12 S (fractions 15−19). In contrast, the larger DHC behaved during sedimentation as a polydisperse system: substantial amounts of this chain were spread throughout the gradient, and discrete protein peaks, corresponding to monomeric or dimeric DHC complexes, were absent. Polydisperse sedimentation of the larger DHC was observed irrespective of gradient ionic strength or preparative regime; variables tested included sucrose gradients prepared in HMEN containing 0, 250, or 500 mM NaCl and dynein prepared by the low ionic strength extraction protocol (data not shown). Furthermore, dynein extracts from ejaculated sperm contained little contaminating tubulin, yet the sedimentation pattern of the larger DHC remained unchanged, which suggests that its polydispersity is not due to association with small microtubule fragments. Thus, it appears that this dynein has a strong tendency to aggregate as does, for example, the

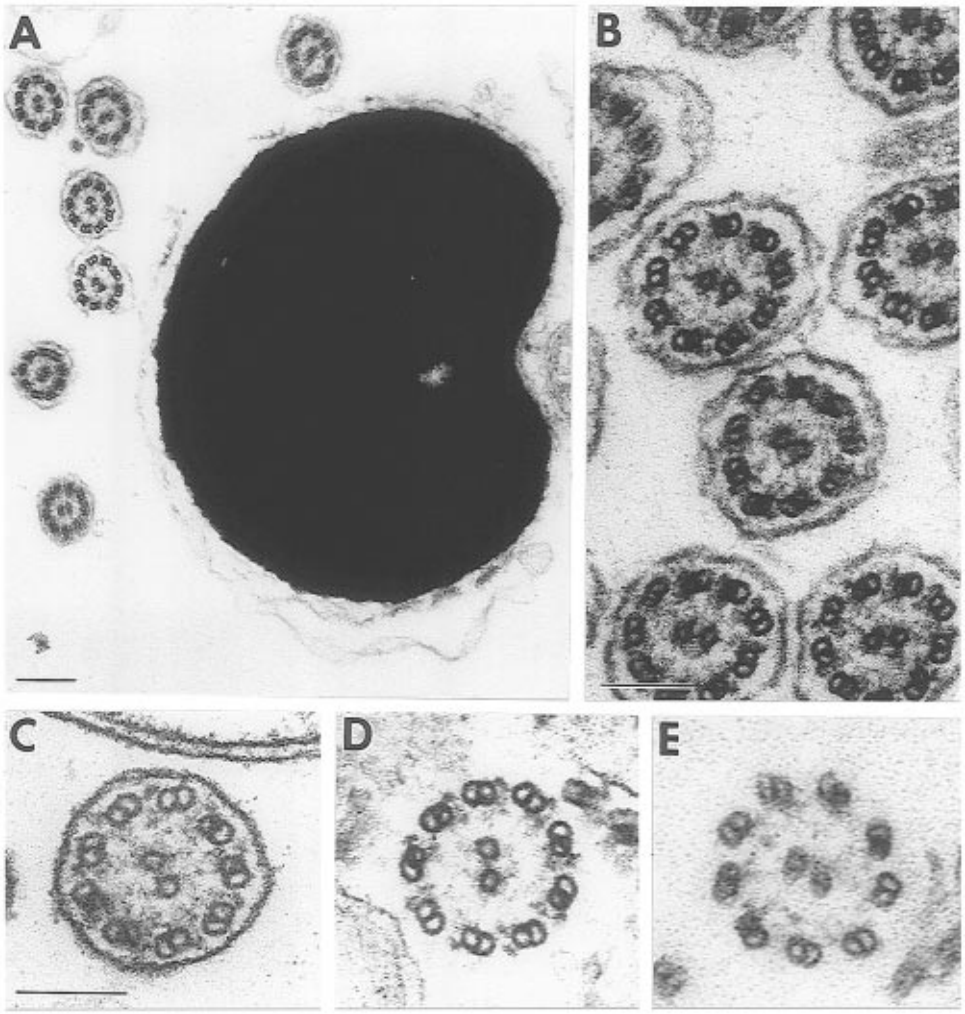

FIGURE 1: Extraction of axonemal dyneins from spermatozoa of *N. coriiceps*: morphological analysis. (A and B) Thin-section electron micrographs of intact testis tissue. (C) Transverse section of isolated sperm flagellum. (D) Transverse section of flagellar axoneme following detergent extraction of flagellar membrane. (E) Transverse section of flagellar axoneme following high-salt/ATP extraction. Bars: (A) 200 nm; (B and C) 100 nm. (C−E) At identical magnification.

α-DHC from sea urchin outer arm dynein (Tang et al., 1982; Moss et al., 1992).

High-resolution electrophoretic analysis of the DHCs sedimenting at ~20 S and at 12 S is shown in Figure 4. At least four discrete DHC bands are evident in the high molecular weight region of the gel. The ~20S dynein fraction contained most of the largest band and a small quantity of one other DHC; the 12S dynein that had been further purified by DEAE ion exchange chromatography (see below) contained mainly the lowest band with minor amounts of the other three. The lowest band was the most refractory to extraction from the axoneme and indeed is the predominant DHC remaining in the post-extraction axonemal sample (Figure 4, lane EA).

Inner Arm Dynein: Subunit Composition and a Heavy-Chain Gene. (A) Composition of the Inner Arm Dynein Complex. The dynein sedimenting at 12 S in sucrose density gradients was further purified by ion exchange chromatography on a Mono Q column [cf. Kagami and Kamiya (1992)]. Silver staining of the purified complex revealed a major DHC which cofractionated with proteins of 43 and 30 kDa (Figure 5). A small amount of a second high molecular weight protein was also detected. This protein pattern is reminiscent of that seen for the I2 and I3 inner arm dyneins from *Chlamydomonas* flagella (Piperno et al., 1990), in which the DHCs are associated with actin, centrin, and p28. Therefore, we tested both axonemes and the purified 12S dynein for the presence of actin (Figure 6) by probing with a monoclonal antibody (C4; ICN Biomedical, Costa Mesa, CA) specific for a highly conserved actin epitope. A single band of approximately 43 kDa was detected in each sample, demonstrating that the 12S dynein species does contain actin. Given its protein profile and actin immunoreactivity, we propose that the 12S dynein (and the Mono Q-purified complex therefrom derived) is an I2/I3-like inner arm species.

(B) An Inner Arm DHC Gene. To investigate further the relationship between Antarctic fish dyneins and those from other organisms, we employed the polymerase chain reaction to amplify from *N. coriiceps* genomic DNA a DHC gene fragment, NcDHCpcr1, that encodes part of the highly conserved region immediately C-terminal to the catalytic (P_1) P-loop. A single product of 247 bp was obtained (Figure 7A). The coding sequence of this product is interrupted by an 87-bp, AT-rich intron flanked by consensus donor and acceptor splice junctions. The predicted peptide sequence is very similar to those of presumptive inner arm DHC isoforms from *Chlamydomonas*, rat, *Paramecium*, *Drosophila*, and sea urchin (Figure 7B) (Gibbons et al., 1994; Wilkerson et al., 1994); for example, the fish and mammalian sequences are 96.3% identical. By contrast, the NcDHCpcr1 peptide sequence is only 63−76% identical to known outer arm DHCs from *Chlamydomonas* and sea urchin. Therefore,

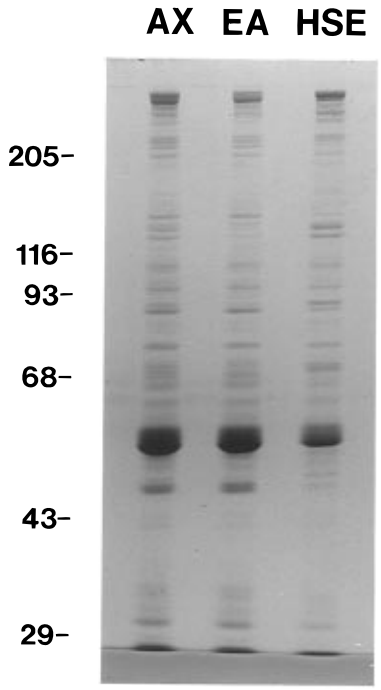

FIGURE 2: Extraction of axonemal dyneins from spermatozoa of *N. coriiceps*: biochemical analysis. Sperm tails from *N. coriiceps* were demembranated with 1% Triton X-100, and the resulting axonemes were extracted with 0.6 M KCl (see Experimental Procedures). Following centrifugation, the high-salt extract was recovered, and the extracted axonemal pellet was resuspended to the original sample volume. Samples (3 μL) of unextracted axonemes (AX), high-salt-extracted axonemes (EA), and the high-salt extract (HSE) were electrophoresed on an 8% SDS−polyacrylamide gel, and the gel was stained with Coomassie Blue. The molecular weights of standards ($\times 10^{-3}$) run on the same gel are indicated on the left.

we conclude that NcDHCpcr1 likely encodes a portion of an inner arm DHC.

To estimate the copy number of this inner arm DHC gene in the genomes of Antarctic fish, we probed Southern replicas of restriction-endonuclease-digested genomic DNA from *N. coriiceps* and from the icefish *C. aceratus* with NcDHCpcr1 (Figure 8). For both species, single bands were observed in several restriction digests, which suggests that there is but one gene for this inner arm DHC isoform in Antarctic fish (see Discussion). Other members of the inner arm DHC gene family were not detected, probably due to the specificity conferred by the intron and the stringent hybridization/wash conditions. Furthermore, for a given enzyme, the sizes of the hybridizing fragments detected in the two fish species are comparable. These observations are consistent with substantial conservation of the coding sequences and the organization of this DHC gene in the two fish genomes.

Outer Arm Dynein: Subunit Composition. Analysis of the outer dynein arm from *N. coriiceps* was complicated by the apparent aggregation of the protein under both high and low ionic strength regimes (see above). Consequently, we employed an immunological approach to identify non-DHC components of the complex. Four monoclonal antibodies originally raised against IC78 and IC69 of the *Chlamydomonas* outer arm (1878A and 1869A, respectively; King et al., 1985, 1986), IC2 of sea urchin outer arm (D9; Ogawa et al., 1990), and IC74 of rat brain cytoplasmic dynein (74-1; Dillman & Pfister, 1994) were used to probe *N. coriiceps* axonemes. In total, five different proteins ranging from 66 to 91 kDa were recognized specifically by the anti-IC antibodies (Figure 9, Table 1). The IC69-specific antibody, 1869A, cross-reacted with proteins of 76 and 88 kDa, whereas the other antibodies each detected single proteins. Table 1 compares the immunologically defined outer arm IC suite of *N. coriiceps* to dynein ICs from four reference species, and Table 2 summarizes the overall protein compositions of outer arm dynein complexes from the rockcod and five other organisms. The DHC/IC content of the *N. coriiceps* outer arm dynein is apparently identical to that from trout, but its LC content remains to be determined. Because

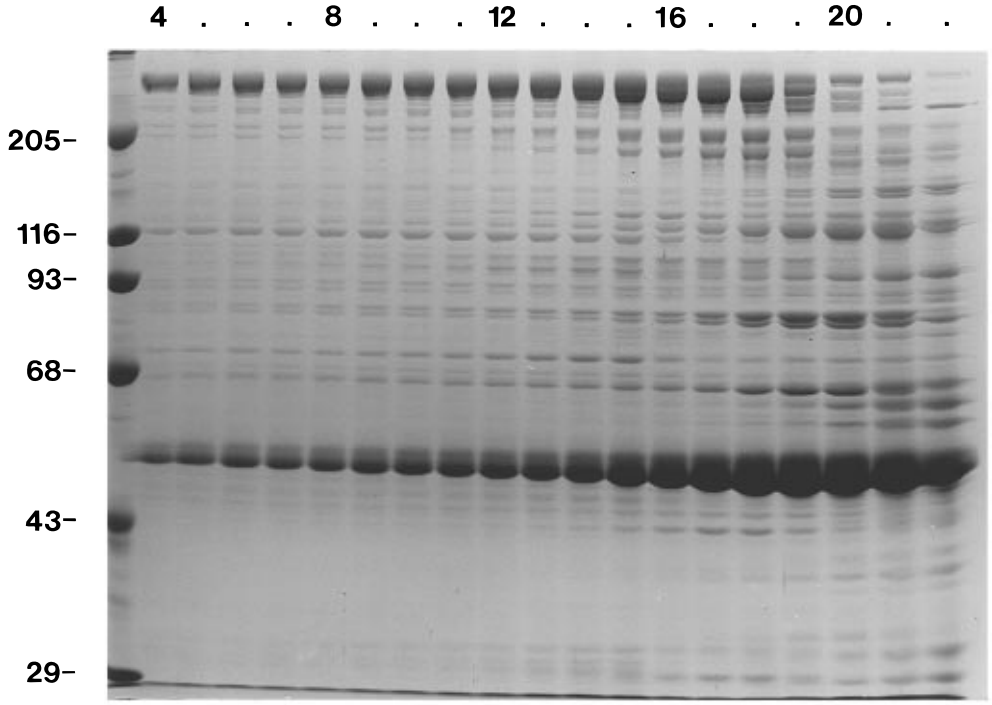

FIGURE 3: Sucrose density-gradient fractionation of the axonemal high-salt extract. The HSE (3 mL at ~1 mg/mL) was sedimented through a 5−25% sucrose gradient (see Experimental Procedures), and fractions of 1 mL were collected. Aliquots (40 μL) of each fraction were electrophoresed on an 8% SDS−polyacrylamide gel, and the gel was stained with Coomassie Blue. Fractions are numbered from the bottom (left) to the top (right) of the gradient. The molecular weights of standards ($\times 10^{-3}$) are indicated on the left.

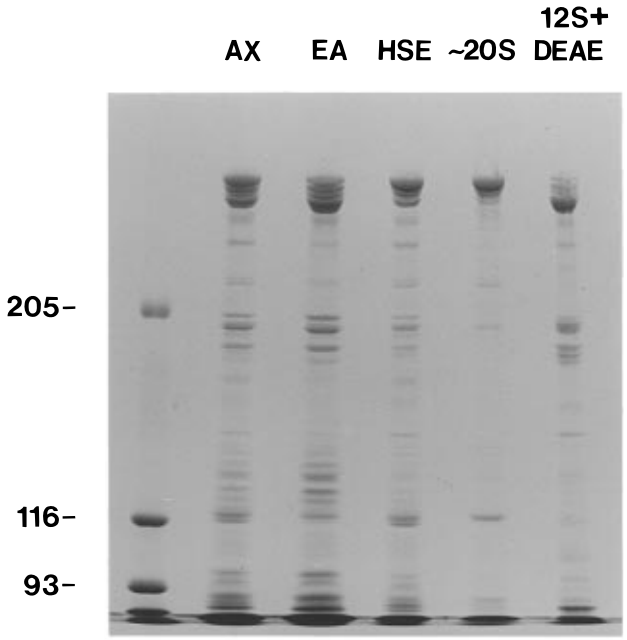

FIGURE 4: High-resolution electrophoretic analysis of dynein heavy chains. Partitioning of the dynein heavy chains during purification was analyzed by electrophoresis of selected samples on a 4 M urea/4% polyacrylamide gel. The gel was stained with Coomassie Blue. Lanes: AX, sperm axonemes (6 μL); EA, salt-extracted axonemes (6 μL); HSE, high-salt extract (6 μL); ~20S, fraction (25 μL) from the 20S region of a sucrose density gradient similar to that shown in Figure 3; 12S + DEAE, 12S dynein peak from a sucrose gradient following further purification on a DEAE-Sephacel column (25 μL). The molecular weights of standards ($\times 10^{-3}$) are indicated on the left.

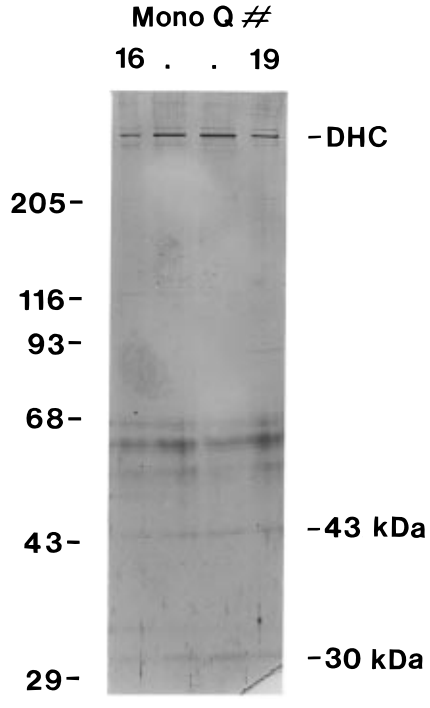

FIGURE 5: Purification of an inner arm dynein complex. Partially purified 12S dynein (e.g., sucrose gradient fractions 15−19, Figure 3) was chromatographed on a Mono-Q column as described under Experimental Procedures. Samples of the dynein-containing peak (Mono-Q fractions 16−19) were electrophoresed on an 8% SDS−polyacrylamide gel, and the gel was stained with silver. The major DHC coeluted with proteins of 43 kDa (possibly actin; see Figure 6) and 30 kDa. The molecular weights of standards ($\times 10^{-3}$) are indicated on the left.

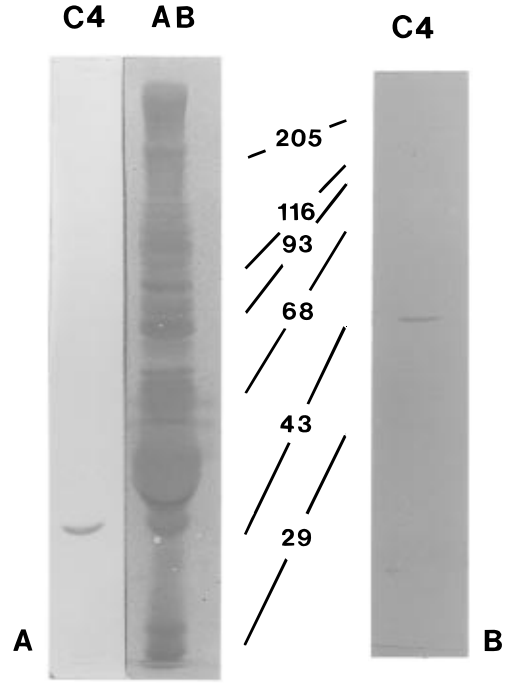

FIGURE 6: Identification of actin in the 12S dynein complex. (A) Axonemal proteins were electrophoresed on an 8% SDS−polyacrylamide gel and then transferred to nitrocellulose by electroblotting. One nitrocellulose strip was stained with amido black to reveal total protein (AB); the adjacent strip was probed with a monoclonal antibody specific for actin (C4). A single immunoreactive band was detected in the axonemal sample. (B) Sucrose gradient-purified 12S dynein was electrophoresed on a 12.5% polyacylamide gel, transferred to nitrocellulose, and probed with the C4 antibody. A single protein of 43 kDa reacted with the antibody. The molecular weights of standards ($\times 10^{-3}$) are indicated between the panels.

many of the ICs from other organisms are related in primary sequence (e.g., IC78 and IC69 of *Chlamydomonas*, IC2 and IC3 of sea urchin, and the single IC of rat cytoplasmic dynein; Mitchell & Kong, 1991; Paschal et al., 1992; Ogawa et al., 1995; Wilkerson et al., 1995), our results suggest that the outer arm dyneins of both cold-adapted and temperate fish contain a family of five related ICs (see Discussion).

Temperature Dependence of Inner and Outer Arm Dynein ATPases. Our ultimate goal is to determine the molecular adaptations that have evolved to maintain the efficient function at low temperatures of the dynein motors of Antarctic fish. As a first step, we have evaluated the temperature dependence of the ATPase activities of *N. coriiceps* inner and outer arm dyneins at temperatures from 0 to 37 °C. The basal, non-microtubule-stimulated ATPase activities of the fish dyneins, although modest in absolute terms, significantly exceeded those of *Tetrahymena* outer arm dynein at temperatures ≤15 °C (at 0 °C, 0.069 and 0.038 μmol of P_i min^{-1} mg^{-1} for inner and outer arm dyneins from *N. coriiceps vs.* 0.009 μmol of P_i min^{-1} mg^{-1} for the *Tetrahymena* enzyme). The inner and outer arms attained their maximal activities (~0.1 μmol of P_i min^{-1} mg^{-1}) at 9 and 19 °C, respectively (Figure 10). Above these temperatures, the ATPase activities of the fish dyneins declined substantially (to 0.050 and 0 μmol of P_i min^{-1} mg^{-1} for inner and outer arms, respectively, at 37 °C), whereas *Tetrahymena* dynein activity continued to increase (e.g., 0.43 μmol of P_i min^{-1} mg^{-1} at 37 °C). The most plausible interpretation of these results is that the greater catalytic efficiencies of the Antarctic fish dyneins at low temperature result from greater

A

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1                                                          60
GTACTGGCAAGACTGAGACCACCAAAGATTTGGCAAAAGCTTTGGCTAAACAGGTAAACC
G  T  G  K  T  E  T  T  K  D  L  A  K  A  L  A  K  Q

61                                                         120
AAAACAGCTTCAGTAAAATACTGACCTGCATTTAGTTTCTTTTTGCTTCTTACAACTCCC

121                                                        180
CTTTTGTTTTTTGTTTCTAGTGTGTGGTGTTCAACTGCTCCGATGGTCTGGACTACAAGG
                     C  V  V  F  N  C  S  D  G  L  D  Y  K

181                                                        240
CCATGAGTAAGTTCTTCAAAGGACTGGCTCAGTCCGGAGCCTGGGCCTGCTTCGACGAGT
A  M  S  K  F  F  K  G  L  A  Q  S  G  A  W  A  C  F  D  E

241 247
TCAACCG
F  N  R
```

B

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                1                                                   54
Nc   DHC1      GTGKTETTKDLAKALAKQCVVFNCSDGLDYKAMSKFFKGLAQSGAWACFDEFNR
Rat  DLP3C     GTGKTETTKDLAKALAKQCVVFNCSDGLDYKAMGKFFKGLAQAGAWACFDEFNR
Par  DHCPD1    GTGKTETVKDLAKALARQCVVFNCSDGLDYKAMGKFFKGLASSGAWSCFDEFNR
Chl  Pcr1      GTGKTETTKDLAKALAKQCVVFNCSDGLDYQAMAKFFKGLASSGAWACFDEFNR
Dro  DHC16F    GTGKTETTKDLAKALAKQCVVFNCSDGLDYKMMGRFFSGLAQCGAWCCFDEFNR
Tg   Dyn7B    GTGKTETSKDLAKAVAKQCVVFNCSDGLDYKAMGKFFKGLAQAGAWACFDEFNR
              ******* ****** * ************  *  ** ***  *** ********
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FIGURE 7: Molecular analysis of an inner arm dynein heavy chain. (A) Nucleotide sequence of the 247-bp PCR product, NcDHCpcr1, amplified from *N. coriiceps* genomic DNA. Perfect copies of the intron donor and acceptor consensus sequences are indicated in bold font. The predicted amino acid sequence is also shown. This sequence has been deposited in the GenBank data base (Accession No. U76502). (B) Comparison between the *N. coriiceps* DHC sequence and DHC sequences from rat (DLP3C; Accession No. D26494), *Paramecium* (DHCPD1; Accession No. L18802), *Chlamydomonas* [pcr1; Wilkerson et al. (1994)], *Drosophila* (DHC16F; Accession No. L23197), and *Tripneustes gratilla* (Dyn7B; Accession No. U03979). The NcDHCpcr1 peptide sequence is 88.9 (*Drosophila*) to 96.3% (rat) identical to the peptides of this reference group. The smallest Poisson probabilities ($P_{(n)}$) for these matches with the *N. coriiceps* sequence range from 1.1×10^{-34} (rat) to 1.0×10^{-31} (*T. gratilla*). Residues conserved in all sequences are indicated by asterisks.

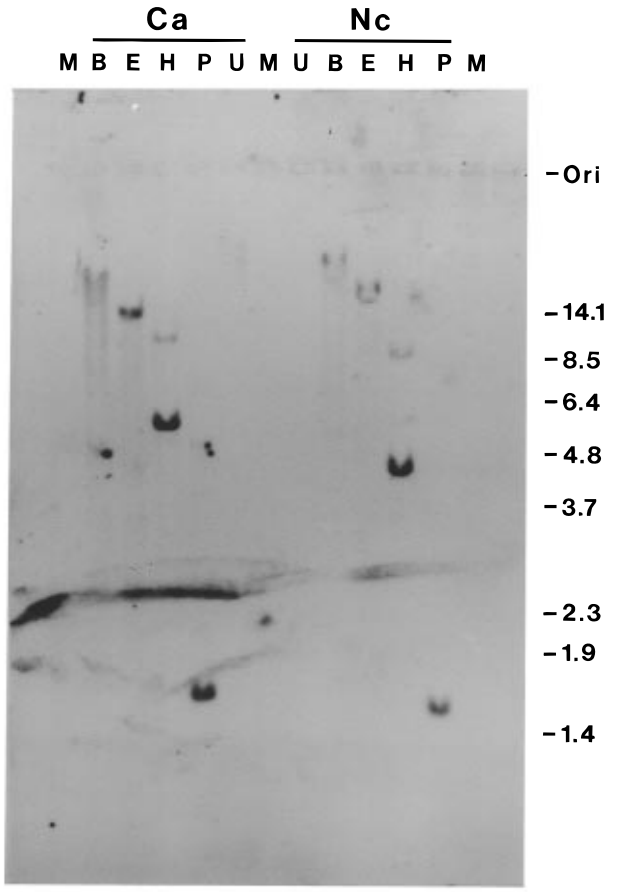

FIGURE 8: Southern hybridization analysis of the inner arm DHC gene. Southern blots of restriction-endonuclease-digested testicular DNAs from two Antarctic fishes, *N. coriiceps* (Nc) and *C. aceratus* (Ca), were probed with the *N. coriiceps* inner arm DHC PCR product (see Experimental Procedures). The DNAs were digested with *Bam*HI, *Eco*RI, *Hin*dIII and *Pst*I (lanes B, E, H, and P, respectively), prior to gel electrophoresis, transfer to a nylon membrane, and chemiluminescent detection of bound probe. Lanes U contained undigested DNA, and lanes M contained DNA standards, the sizes of which are indicated at right (kb). With the exception of the *Hin*dIII digests, single DNA fragments were detected, which suggests that the rockcod and icefish genomes each contain one gene for this DHC.

flexibility in the active site(s) of their protein subunits, which also renders the complexes more susceptible to denaturation at elevated, nonphysiological temperature.

DISCUSSION

Although flagellar dyneins from many organisms have been described, most studies have concentrated on the functional and structural properties of the outer arms. This has been particularly true in vertebrate species, from which, until this report, inner arm dyneins had not been purified in significant quantity. Here we report the isolation, purification, and characterization of both inner and outer arm dyneins from sperm flagella of an Antarctic rockcod, *N. coriiceps*. The inner arm dynein sediments as a discrete, 12S complex, and its subunit composition is similar to that of the inner arm dyneins of *Chlamydomonas*. Furthermore, one inner arm DHC isoform appears to be encoded by a single-copy gene. The outer arm complex, which demonstrates a propensity to aggregate, is similar in composition to the outer arm dynein of trout sperm flagella (Gatti et al., 1989; King et al., 1990). The basal ATPase activities of both dyneins at physiological temperature (0 °C) are substantial, consistent with cold adaptation of motor function. Thus, the flagellar dyneins of *N. coriiceps* provide the opportunity, within a single vertebrate species, to examine the functional and regulatory properties of both *inner* and *outer* arms and to analyze the molecular basis of cold adaptation of microtubule motors.

Inner Arm Dynein. The sedimentation properties and polypeptide composition of the *N. coriiceps* inner arm complex suggest that it corresponds to the I2 or I3 inner arms of *Chlamydomonas* (Piperno et al., 1990). The fish complex and algal I2/I3 inner arms (Goodenough & Heuser, 1984; Smith & Sale, 1991) both sediment at 11−12 S as single-headed dynein particles. Furthermore, both actin and p28 (estimated at 30 kDa for *N. coriiceps*) appear to be associated with two electrophoretically rapid DHCs, one major and one minor. Thus, we propose that this vertebrate I2/I3 homolog is likely to be arranged along the axoneme and to interact with the dynein regulatory complex as do the I2/I3 inner arms of *Chlamydomonas* (Mastronarde et al., 1992; Piperno et al., 1992).

The DHCs of protistans and invertebrates are encoded by small gene families that can be subdivided into inner arm, outer arm, and cytoplasmic isoform classes (Gibbons et al., 1994; Wilkerson et al., 1994). Using PCR and Southern analysis, we have detected an inner arm DHC gene in *N. coriiceps* (and *C. aceratus*) that appears to belong to isoform class 7 (Gibbons et al., 1994). Whether this, or another, gene encodes the DHC polypeptide(s) of the *N. coriiceps* inner arm I2/I3-like complex remains to be determined. The intron present in the inner arm gene fragment, NcDHCpcr1, is striking in its high content of AT base pairs. AT-rich introns

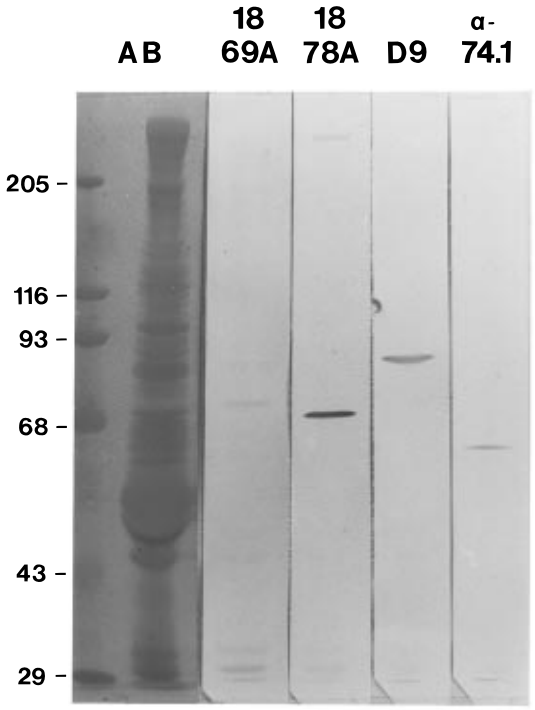

FIGURE 9: Immunological analysis of *N. coriiceps* outer arm dynein ICs. Axonemal proteins were separated on an 8% SDS−polyacrylamide gel and then transferred to nitrocellulose. One strip was stained with amido black (AB) to reveal total protein. The remaining lanes were probed with monoclonal antibodies specific for dynein ICs. Antibodies 1878A, D9, and 74-1 each recognized single protein bands (73, 91, and 66 kDa, respectively). Antibody 1869A reacted less strongly with two proteins of 88 and 76kDa. The two low molecular weight bands visible in these blots result from reaction with the secondary antibody. The molecular weights of standards ($\times 10^{-3}$) are indicated at the left.

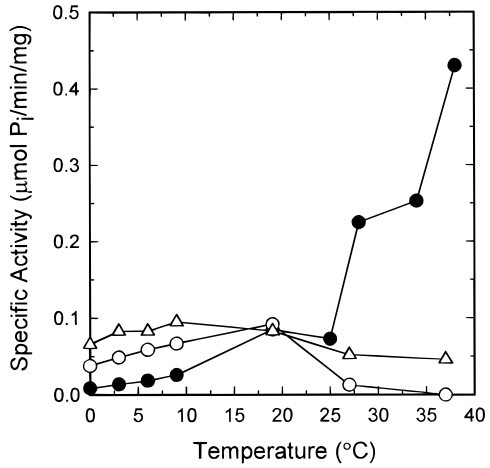

FIGURE 10: Temperature dependence of inner and outer arm dynein ATPases. The specific ATPase activities of *N. coriiceps* flagellar inner arm (Δ) and outer arm (○) dyneins and of the 22S ciliary outer arm dynein from *Tetrahymena* (●) were determined over the temperature range 0−37 °C. *N. coriiceps* samples containing predominantly inner arm dynein and outer arm dynein were obtained from the 12S and 20S regions of sucrose density gradients. Enzyme activity is expressed in units of micromoles of phosphate released per minute per milligram of protein. Standard errors of the mean associated with the points ($n = 3-6$ measurements per datum) are smaller than the graph symbols.

have been observed in other Antarctic fish genes, which led Vayda et al. (1995) to hypothesize that the low melting temperatures of these regions may be an adaptation to facilitate DNA strand separation during replication and transcription at low temperature.

Table 1: Immunoreactivity of Outer Arm Dynein ICs

	organism				
antibody	*N. coriiceps*[a]	*Chlamydomonas*[a]	rat[b]	sea urchin[c]	trout[d]
1869A	88 & 76 kDa	IC69	nk[e]	IC3	IC2
1878A	73 kDa	IC78	nk	−	IC2
D9	91 kDa	nk	nk	IC2	nk
74-1	66 kDa	nk	IC74	nk	nk

[a] King et al. (1985). [b] Dillman & Pfister (1994). [c] Ogawa et al. (1990). [d] King et al. (1990). [e] nk, not known.

Table 2: Composition of Outer Arm Dyneins

source	DHCs	ICs	LCs	reference
N. coriiceps sperm flagella	2	5[a]	?	this work
trout sperm flagella	2	5	6	Gatti et al. (1989)
porcine respiratory cilia	2	2	?	Hastie et al. (1988)
sea urchin sperm flagella	2	3	4	Bell et al. (1979)
Chlamydomonas flagella	3	2	8	Pfister et al. (1982)
Tetrahymena cilia	3	2	4	Porter & Johnson (1983)

[a] Based on immunological analysis.

Outer Arm Dynein. The *N. coriiceps* outer arm dynein complex, like that of trout, contains two DHCs and five related ICs. Thus, the quaternary structural organization of the *N. coriiceps* outer arm particle is likely to be similar to that of the temperate fish. Particularly noteworthy are the large IC families of the fish outer arms (Table 2); perhaps this is a structural feature unique to the piscine taxon. Not yet understood is the propensity of the *N. coriiceps* outer arm to aggregate under solution conditions that yield the trout outer arm as a discrete, 19S species. Although this phenomenon could result indirectly from the molecular changes necessary to adapt the fish enzyme to low temperature function, outer arm dyneins from temperate organisms [e.g., the outer arm α-DHC of the sea urchins *Tripneustes gratilla* (Tang et al., 1982) and *Strongylocentrotus purpuratus* (Moss et al., 1992)] have also been reported to aggregate.

Temperature Dependence of Dynein ATPase Activities. The basal ATPase activities of *N. coriiceps* inner and outer arm dyneins exceed substantially that of a mesophilic referent, 21S outer arm dynein from *Tetrahymena*, at low temperatures. The inner arm and outer arm enzymes attain their maxima (∼0.1 μmol of P_i min^{-1} mg^{-1}) at 9 and 19 °C, respectively, and their activities decrease rapidly at higher temperatures. The higher catalytic efficiencies of the psychrophilic enzymes at low temperatures (Figure 10) suggest that the free energy necessary to attain the transition state ($\Delta G^{\ddagger}$, the free energy of activation) has been reduced by evolution of greater polypeptide flexibility at or near their active sites [cf. Hochachka and Somero (1984)]. The tradeoff associated with this increased flexibility is greater susceptibility to thermal denaturation at higher, mesophilic temperatures (∼20 °C). As temperature increases, then, loss of local (i.e., active site) and long-range native structure by the psychrophilic *N. coriiceps* enzymes offsets the catalytic rate increases expected from a more reactive substrate population, which leads to activity loss above the "optimal" temperatures of ∼10−20 °C. The more rigid *Tetrahymena* outer arm presumably experiences less denaturation in the temperature range examined, which results in a monotonic increase in ATPase activity with increasing temperature.

The optimal strategy for analysis of temperature adaptations in *N. coriiceps* dyneins requires comparison of their

activities to those of dyneins from related, but mesophilic, fishes. Perhaps the best phylogenetic referent would be the New Zealand black cod, *Notothenia angustata*, a temperate congener (body temperature range 6−15 °C) of *N. coriiceps* with similar morphology and life style, but studies of axonemal dyneins from this species are lacking. At present, such comparison is limited to the trout *S. gairdneri*, a salmonid fish rather distantly related to the Antarctic notothenioids. The ATPase activity of the 19S outer arm dynein from the trout, measured at approximately 22 °C, is 1.1 ± 0.3 μmol of P_i min^{-1} mg^{-1} (Gatti et al., 1989), which is approximately 10-fold larger than the activity of *N. coriiceps* outer arm dynein at 19 °C and some 25-fold larger than the activity of the rockcod enzyme at 0 °C. However, direct comparison of these values is rendered problematic both by the apparent denaturation of the *N. coriiceps* enzyme at elevated temperature and by its tendency to aggregate, which may perturb the ATPase activity. Thus, meaningful assessment of the degree of cold adaptation of *N. coriiceps* outer arm dynein relative to the trout homolog will require, at a minimum: (1) determination of the complete thermal profile (0−37 °C) of the *S. gairdneri* outer arm ATPase activity; and (2) development of extraction conditions that prevent aggregation of the *N. coriiceps* outer arm followed by reanalysis of its activity profile.

Summary. In this report, we have described a fish system, the Antarctic rockcod *N. coriiceps*, which should serve as a useful model for structural and functional studies of both the inner arm and the outer arm dyneins of vertebrates and for analysis of cold adaptation of microtubule motor function. Its advantages include the availability of large quantities of sperm during the spawning season and the ease of purification of dyneins in milligram amounts. Of particular importance, this system provides the first example of a vertebrate inner arm dynein, one that appears to correspond to the I2 and/or I3 inner arms of *Chlamydomonas*. Our work, and the results of Gatti et al. (1989), highlight the utility of fish systems for analysis of dynein motors in vertebrate taxa.

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