Anhydroretinol and 14-hydroxy-4,14-retro-retinol, retro-retinoids endogenous to both mammals and insects, act as agonist and antagonist, respectively, in controlling proliferation in lymphoblasts and other retinol-dependent cells. We describe here the identification, purification, cloning, and bacterial expression of the enzyme retinol dehydratase, which converts retinol to anhydroretinol in Spodoptera frugiperda. Retinol dehydratase has nanomolar affinity for its substrate and is, therefore, the first enzyme characterized able to utilize free retinol at physiological intracellular concentrations. The enzyme shows sequence homology to the sulphotransferases and requires 3'-phosphoadenosine 5'-phosphosulfate for activity.

**Experimental Procedures**

Cell Lines—Sf-21 and Sf-9 were a gift from Dr. Philip W. Majerus (Washington University, St. Louis, MO). The cells were grown in Grace's insect medium/10% fetal calf serum at 26 °C.

Retinols and Other Reagents—All-trans-retinol, PAPS, and other chemicals were purchased from Sigma. [3H]Retinol was purchased from DuPont NEN. Synthetic AR was synthesized by acid-catalyzed dehydration of all-trans-retinol, followed by purification on HPLC as described (11).

Retinol Dehydratase—Ten g of Sf-21 cell pellet were sonicated to greater than 95% lysis in the presence of protease inhibitors. Cellular debris was removed by centrifugation (10,000 × g for 10 min), and a high speed supernatant (100,000 × g for 60 min) was prepared. The high speed supernatant (20 ml) was applied to (a) an Ultragel AcA54 gel filtration column (LKB, Rockland, MD) (4 × 100 cm; flow rate, 0.6 ml/min; 20 mM Tris, pH 7.5; fraction volume, 7 ml). Fractions corresponding to an apparent size of 35-45 kDa were pooled and applied in 1.5 mM (NH₄)₂SO₄ to (b) an HIC Econopak column (Bio-Rad, 5-ml bed volume; flow rate, 1 ml/min; 20 mM Tris·HCl, pH 7.5; linear gradient, 1.0-5.0 mM NaCl in 50 ml). Active fractions between 0.7 and 0.4 mM (NH₄)₂SO₄ were dialyzed overnight against 20 mM Tris·HCl, pH 7.5, and applied to (c) a MonoQ Econopak column (Bio-Rad) (5-ml bed volume; flow rate, 1 ml/min; 20 mM Tris·HCl, pH 7.5; linear gradient, 0-1.0 mM NaCl in 60 min). Active fractions eluting between 0.2 and 0.3 mM NaCl were diluted 10-fold with distilled water, and loaded onto (d) a DEAE-SPW column (Waters, Bedford, MA) (1 × 5 cm; flow rate, 1 ml/min; 20 mM Tris·Cl, pH 7.5; linear gradient of 0-50 mM NaCl in 5 min, 0.1-0.2 mM in 35 min, and 0.2-1.0 mM in 35 min). Retinol dehydratase activity was assayed in column fractions (10-40-μl aliquots complemented with 50 μl of Sf-9 cytosolic supernatant) as described above. Proteins were analyzed on 10% acrylamide SDS-polyacrylamide gel electrophoresis gels under reducing conditions and visualized by Coomassie or silver staining.

Cloning and Expression of Retinol Dehydratase—Purified retinol dehydratase was electroblotted onto nitrocellulose membrane. The protein sequencing facility of Sloan-Kettering Institute was used to subject the protein to trypsin digestion. Peptides were separated by reverse-phase HPLC. Three peptides were sequenced, and the predicted masses were confirmed by mass spectroscopy. Degenerate oligonucleotides were synthesized to the peptides, and a 200-bp probe was generated from Sf-21 cDNA by PCR. The 32P-labeled 200-bp DNA probe was used to screen a Sf-21 cDNA pSPORT-1 expression library (Life Technologies, Inc.). The cDNA sequence was determined from both strands. The coding region of a full-length clone (#61) was subcloned into the bacterial expression vector PET-15b (Novagen, Madison, WI). Recombinant enzyme was purified using a Ni²⁺-affinity column.

Kinetic Analysis—Kinetic studies were performed with native retinol dehydratase (0.1 μg/assay) and [3H]retinol in the presence of constant substrate carrier protein concentrations, either delipidated BSA or recombinant CRBP at 24 °C. The initial rate of AR synthesis at different substrate concentrations was determined by on-line liquid scintillation counting of [3H]AR after HPLC separation. Conversion of...
(\textsuperscript{3}H) retinol was less than 10\% of the total at the end point of each assay. The concentration of free retinol present in each assay was calculated based on published values for the dissociation constant of the relevant carrier protein (BSA \(K_d = 1 \times 10^{-6} \text{ M}\); CRBP \(K_d = 1.2 \times 10^{-8} \text{ M}\)). Data were analyzed with the computer program k.cat (Biometallics, Inc., Princeton, NJ).

Fluorimetric Titration of Retinol Dehydratase—Fluorimetric titrations were conducted according to the method of Cogan et al. (18–21). Recombinant retinol dehydratase (1 \(\mu\text{M}\)) was titrated with retinol from a concentrated solution in ethanol (final ethanol concentration, \(<2.0\%\)).

Binding was monitored by following the fluorescence enhancement of retinol (excitation, \(A = 330 \text{ nm}\); emission, \(A = 480 \text{ nm}\); Fig. 4B, ○). The fluorescence contribution of free retinol was determined by titration with retinol in the absence of protein (Fig. 4B, □), analyzed by linear regression analysis (Fig. 4B, ---) and subtracted from the titration data to give the corrected titration binding curve (Fig. 4B, ●). The data were fitted (Fig. 4B, ———) using an equation derived from simple binding theory (23).

**RESULTS AND DISCUSSION**

AR Is Enzymatically Produced in Sf-21 Cells—The all-trans and cis isomers of AR (10, 11) were identified as the predominant metabolites of \textsuperscript{3}H retinol in the \textit{S. frugiperda} cell line Sf-21 (Fig. 1A); the closely related cell line Sf-9, however, did not synthesize detectable levels of AR (Fig. 1B). AR isomers were identified by coelution with synthetic standards and their characteristic vibronic fine structure in the UV-visible absorption spectrum: maxima at 270, 348, 368, and 390 nm.

Purification of retinol dehydratase activity solely in the 100,000 \(\times\) g supernatant fraction (Fig. 1, C and D). Enzyme activity was lost following dialysis (Fig. 1E) but could be restored by supplementation with cytosol from either Sf-9 cells (Fig. 1F) or heat-inactivated Sf-21 cells (data not shown), indicating a requirement for a small dialyzable cofactor.

The enzyme activity was purified by sequential column chromatography. Activity was monitored by quantitation of \textsuperscript{3}H AR production in the presence of Sf-9 cytosolic factor. The activity eluted in one peak, corresponding to an apparent size of 35–45 kDa from a gel filtration column, at 0.7–0.4 \(\text{ M}\) ammonium sulfate from a methyl hydrophobic interaction column, at 0.2–0.3 \(\text{ M}\) NaCl from a Mono Q anion exchange column, and at 0.125 \(\text{ M}\) NaCl from a DEAE-5PW column fractionation. The final enrichment of the enzyme activity was 11,700-fold and correlated with a 41-kDa protein (Fig. 2A). Amino acid sequence information on three tryptic peptides (peptide sequences bracketed in Fig. 3) was used to generate a specific cDNA PCR probe for the screening of a Sf-21 cDNA plasmid library. Four independent full-length clones predicted the same 352-amino acid residue protein (41.5 kDa). Each clone exhibited retinol dehydratase activity when assayed in crude sonicates of transformant bacterial cultures. Top panel, pSPORT1 vector control; bottom panel, full-length clone pSPORT1-RDHYDRT 61 expressed in DH10B. C, SDS-polyacrylamide gel electrophoresis of HiTag-purified active recombinant retinol dehydratase (lane 1). Lane 2, protein size markers.
amino acid sequence (Fig. 3) is homologous to that of sulfotransferases (overall 20–26% amino acid homology; 35% for a contiguous 200-amino acid C-terminal region). Sulfotransferases transfer sulfonate (SO$_3^-$) groups from the universal active sulfate donor to acceptor alcohol or amine functional groups. Using purified recombinant retinol dehydratase, PAPS was necessary to restore activity and was sufficient in replacing the Sf-9 cytosol supplement (\(k_m = 0.26 \pm 0.05 \mu M; n = 3\)), whereas adenosine 5'-phosphosulfate, the biosynthetic precursor of PAPS, was inactive. The reaction mechanism for retinol dehydratase probably proceeds via the sulfated intermediate, retinyl sulfate.

**Kinetic Analyses**—The \(k_m\) for retinol was determined by kinetic analyses (Fig. 4A), performed with purified natural retinol dehydratase and confirmed with recombinant enzyme. Carrier protein for retinol, either delipidated BSA or recombinant human CRBP-1, was added to reduce nonspecific binding and micelle formation. All assays gave a \(V_{max}\) of 490 ± 50 pmol AR min$^{-1}$ mg$^{-1}$ enzyme (n = 5), but the \(k_m\) for retinol varied between assays containing different carrier proteins when the total retinol present in the system was used as the nominal substrate concentration (\(k_m = 2.6 \pm 0.05 \mu M; n = 3\)), whereas adenosine 5'-phosphosulfate, the biosynthetic precursor of PAPS, was inactive. The reaction mechanism for retinol dehydratase probably proceeds via the sulfated intermediate, retinyl sulfate.

**Binding of Retinol to Retinol Dehydratase**—The extremely low \(k_m\) value for retinol suggests a high affinity interaction between enzyme and substrate. Direct binding experiments performed with recombinant enzyme by fluorescence titrations in the absence of cofactor PAPS, i.e. under conditions that do not allow catalysis, indicated an equilibrium dissociation constant (\(K_d\)) of 2.7 nm ± 0.8 nm (n = 7) (Fig. 4B). These data also indicate that high affinity binding of retinol to the enzyme does not require the presence of the cofactor PAPS. Due to its high
affinity, retinol dehydratase would not be rate limited at physiological concentrations of free retinol.

Distinct sulfotransferases are responsible for the sulfation of steroid hormones, thyroid hormones, monoamine neurotransmitters, and alcohols (25); sulfation of these bioactive signaling molecules modulates their activities (26). Sulfotransferases are also responsible for detoxification of xenobiotics by sulfation (25, 26). However, retinol dehydratase does not appear to act as part of a xenobiotic elimination pathway. The low K_d and K_m of retinol dehydratase for retinol (10^{-9} \text{M}) is indicative of a highly specific interaction similar to endogenous steroid substrates and their cognate sulfotransferases. In contrast, xenobiotic substrates and the aryl sulfotransferase family members generally display 10^{-3}–10^{-5} \text{M} affinities (27, 28). Furthermore, insects utilize retinol metabolites (retinol and 3-OH-retinol) as chromophores in vision, demonstrating a requirement for retinol-metabolizing enzymes. In addition to AR, insect cell lines utilizing enzyme may regulate local levels of "vitamin A activity" in target cells and tissues.

We speculate that in mammals the synthesis of the antagonist AR, from the prohormone agonist retinol via the retinol dehydratase (sulfotransferase) pathway, may help to locally inhibit the proliferation of fibroblasts and suppress immune system activation. This may be of benefit in tissues continually subjected to environmental stress and insult, e.g. liver and lung, where chronic nonspecific activation needs to be down-regulated; both tissues are sites of high AR biosynthetic activity.\textsuperscript{3} The enzymatic control of ligand synthesis, in addition to putative retro-retinoid receptor proteins, is, therefore, likely to be an important regulatory element in this novel signal transduction pathway. Retinol dehydratase represents the prototypic experimental system for studying how a cytosolic retinol-utilizing enzyme may regulate local levels of "vitamin A activity" in target cells and tissues.

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\textsuperscript{2} F. Grün, U. Hämmerling, and J. Buck, unpublished results.

\textsuperscript{3} F. Grün and J. Buck, unpublished results.

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