Universitat Autònoma de Barcelona
Departament de Bioquímica i Biologia Molecular

# PAPER DE LES ALDO-CETO REDUCTASES EN EL METABOLISME DE RETINOIDES:

## ESTUDI FUNCIONAL I ESTRUCTURAL DE L'AKR1B10

Tesi presentada per adquirir el grau de Doctor en Bioquímica i Biologia Molecular per
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Treball realitzat en el Departament de Bioquímica i Biologia Molecular, Unitat de Bioquímica de la Facultat de Ciències, de la Universitat Autònoma de Barcelona, sota la direcció dels doctors
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Per sort, durant aquests anys he pogut treballar amb molta gent diferent, i en molts llocs diferents. Totes aquestes persones saben de sobres que els estic molt agraït, doncs així els hi he dit en nombroses ocasions. Ara, però, tinc l'oportunitat de dir-ho per escrit i en públic: Moltes gràcies a tots i totes!

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MOLTES GRÀCIES A TOTS!!!

# ÍNDEX

**ABREVIATURES**

| | |
|---|---|
| ADH | alcohol deshidrogenasa |
| AKR | aldo-ceto reductasa |
| ALDH | aldehid deshidrogenasa |
| AR | aldosa reductasa |
| ARAT | acil-CoA:retinol acil transferasa |
| ARI | inhibidor de l'AKR1B1 |
| BSA | albúmina de sèrum boví |
| CRABP | proteïna cel·lular unidora d'àcid retinoic |
| CRBP | proteïna cel·lular unidora de retinol |
| DMEM | *Dubelcco's modified Eagle's medium* |
| DPPC | α-dipalmitoil fosfatidil colina |
| HCC | hepatocarcinoma cel·lular humà |
| HPLC | cromatografia líquida d'alta resolució |
| HSD | hidroxiesteroide deshidrogenasa |
| kDa | quilodalton |
| $k_{cat}$ | constant catalítica |
| Ki | constant d'inhibició |
| $K_m$ | constant de Michaelis |
| LRAT | lecitina-retinol acil transferasa |
| MDR | deshidrogenasa/reductasa de cadena mitjana |
| NAD+/NADH | dinucleòtid de nicotinamida – adenina (oxidat/reduït) |
| NADP+/NADPH | dinucleòtid fosfat de nicotinamida – adenina (oxidat/reduït) |
| NSCLC | carcinoma de pulmó de cél•lules no petites |
| RAR | receptor d'àcid retinoic |
| RARE | element de resposta del DNA a àcid retinoic |
| RBP | proteïna unidora de retinol |
| RDH | retinol deshidrogenasa |
| REH | hidrolasa d'èsters de retinol |
| RXR | receptor d'àcid 9-cis-retinoic |
| SDR | deshidrogenasa/reductasa de cadena curta |
| SDS | dodecil sulfat sòdic |
| U | unitat internacional d'activitat enzimàtica |
| ε | coeficient d'absoció molar |
| λ | longitud d'ona |

**RESUM**

El nostre grup havia descrit l'única alcohol deshidrogenasa (ADH) de vertebrats amb especificitat per NADP(H): l'ADH8 d'amfibis (Peralba *et al.,* 1999). Tot i que aquest enzim també presentava activitat etanol deshidrogenasa, va mostrar una clara preferència per a la reducció d'aldehids, com per exemple el tot-trans-retinaldehid. Per establir la distribució de l'enzim en altres grups de vertebrats, es va iniciar la recerca del seu homòleg en aus. Es va identificar en pollastre un proteïna monomèrica de 36 kDa, amb propietats cinètiques similars a les de l'ADH8. Aquest enzim, però, va resultar ser un nou membre de la superfamília de les aldo-ceto reductases (AKR), la AKR1B12 (Crosas et al., 2001). Per primer cop, s'havia identificat una AKR activa amb retinoides. Atès que l'AKR1B12 presenta una identitat seqüencial pròxima al 70% amb l'AKR1B1 i l'AKR1B10 humanes, es va decidir ampliar l'estudi de l'activitat retinoide oxidoreductasa entre les AKR.

En la present Tesi Doctoral, hem aprofundit en l'estudi de l'activitat retinal reductasa de membres de la superfamilia de les aldo-ceto reductases (AKR). El treball es va iniciar amb la caracterització in vitro de l'activitat amb retinoides d'AKR humanes de la família AKR1. En col•laboració amb el grup de la Dra. Natalia Kedishvili (actualment en el Department of Biochemistry and Molecular Genetics, Schools of Medicine and Dentistry, University of Alabama at Birmingham, EUA) es va realitzar una anàlisi comparativa de l'activitat retinol oxidoreductasa d'enzims de les superfamílies de les deshidrogenases/reductases de cadena mitjana (MDR), deshidrogenases/reductases de cadena curta (SDR) i AKR. Es va emprar un nou mètode basat en la solubilització de retinoides amb BSA, i anàlisi mitjançant HPLC. Així, es van poder determinar els valors de les constants cinètiques per a enzims humans de les tres superfamílies sense l'efecte d'inhibició del Tween-80, detergent utilitzat tradicionalment per a l'estudi cinètic de les MDR i AKR (Capítol I). Aquests resultats van posar de relleu que el valor de $K_m$ per a retinoides és similar (0,1-1 μM) en els enzims estudiats. Les principals diferències es centren en el valor de $k_{cat}$, el qual va variar fins a tres ordres de magnitud entre els diferents enzims. Un altre resultat destacable, va ser la desmostració que cap dels enzims era capaç d'utilitzar com a substrat retinol, o retinal, unit a la proteïna cel•lular unidora de retinol (CRBPI). Fins el present treball, s'utilitzaven dos arguments per defensar un paper principal de les SDR en l'oxidació de retinol a retinal, primera etapa de síntesi de l'àcid retinoic: 1) Valors de $K_m$ inferiors de les SDR per als retinoides (fins a dos ordres de magnitud respecte les ADH). 2) Capacitat de reconèixer l'holoCRBPI com a substrat, una propietat aparentment exclusiva

d'algunes SDR. El nostre estudi, a part de descartar aquests dos arguments, ha introduït les AKR com a tercer grup enzimàtic implicat en el metabolisme de retinoides.

La segona part de la Tesi es centra en l'estudi estructural i funcional de l'AKR1B10, l'AKR que presenta una major eficiència catalítica per a la reducció del retinal. Aquest enzim és sobrexpressat en diferents tipus de càncers humans, el que va fer augmentar el nostre interès per determinar el seu paper en la ruta de síntesi de l'àcid retinoic. A part de l'estudi in vitro de l'activitat retinoide oxidoreductasa de l'enzim, també es va dur a terme un estudi in vivo. Utilitzant cèl•lules COS-1 com a model, i mitjançant transfecció transitòria, es va poder observar que AKR1B10 també participava en la reducció de retinal in vivo, però que en canvi no era capaç d'oxidar retinol.

Finalment, i en col•laboració amb el grup de cristal•lografia de proteïnes del Dr. Ignasi Fita (Parc Científic de Barcelona-CSIC), es va obtenir l'estructura tridimencional del complex ternari AKR1B10-NADP+-tolrestat. El tolrestat és un inhibidor d'AKR1B1 àmpliament estudiat com a fàrmac en el tractament de les complicacions de la diabetis, però que també inhibeix AKR1B10 tant in vitro com in vivo. Tot i l'elevada identitat seqüencial entre AKR1B1 i AKR1B10, aquests enzims presenten constants cinètiques molt diferents per a la reducció del retinal. Així, gràcies a la resolució de l'estructura d'AKR1B10, esperem poder estudiar els determinants estructurals que confereixen a cada enzim les seves propietats cinètiques característiques, així com establir les bases per al disseny de nous inhibidors específics per a cadascun d'ells.

# INTRODUCCIÓ GENERAL

## 1 Aldo-ceto reductases

Les aldo-ceto reductases (AKR) constitueixen una de les superfamílies enzimàtiques de la classe de les oxidoreductases. Tradicionalment, la majoria d'oxidoreductases d'àmplia especificitat estudiades han format part de les superfamílies de les deshidrogenases-reductases de cadena curta (SDR) i les deshidrogenases-reductases de cadena mitjana (MDR). En la darrera dècada, però, s'ha descrit un gran nombre de noves oxidoreductases no relacionades amb les dues superfamílies esmentades, també d'especificitat poc estricte, sobretot amb activitat de reducció de grups carbonils. Aquests enzims, molt similars entre si, i que han estat descrits en vertebrats, invertebrats, plantes, protozous, fongs i bacteris, formen part de les AKR (Jez and Penning 2001). De fet, no va ser fins a finals dels anys 80 que es va utilitzar el terme AKR per definir la superfamília (Bohren *et al.,*, 1989).

### 1.1 Propietats cinètiques i estructurals de les AKR

Les AKR són enzims dependents de NAD(P)(H). Tot i que es tracta d'enzims amb seqüències altament conservades, petites variacions en els llaços de l'extrem C-terminal i substitucions puntuals en el centre actiu els confereixen una alta variabilitat en l'especificitat de substrat, de manera que poden reduir una gran varietat de grups carbonil de carbohidrats, esteroides, isoflavonoides, i compostos aromàtics i alifàtics, (Jez *et al.,* 1997).

**Aldehid o Cetona + NAD(P)H + $H^+$**

$\uparrow\downarrow$ AKR

**Alcohol + $NAD(P)^+$**

En general, les AKR segueixen un mecanisme seqüencial ordenat bi-bi, en què primer s'uneix el cofactor, i l'etapa limitant de velocitat és l'alliberació del cofactor oxidat (Wilson *et al.,* 1992; Jez *et al.,* 1997).

Les AKR són monòmers amb uns 320 residus d'aminoàcid i uns 36 kDa de pes molecular. Presenten una tètrada catalítica característica: Asp44, Tyr49, Lys78, i His111 (Jez *et al.,* 1997). A diferència de les altres dues superfamílies d'oxidoreductases, no utilitzen el plegament de Rossmann en el domini d'unió del cofactor, sinó el barril $(\alpha/\beta)_8$, que constitueix un motiu molt conservat i en què el NAD(P)H es situa en el seu interior. La unió del substrat es dóna

principalment a través dels llaços situats en una de les cares del barril $(\alpha/\beta)_8$, on es troben la majoria de les variacions entre les diferents AKR (Figura 1). Principalment es diferencien 3 llaços: El llaç A, que va del residu 112 al 136; el llaç B, que es troba entre els residus 216 i 227; i el llaç C, entre els residus 299 i 310.

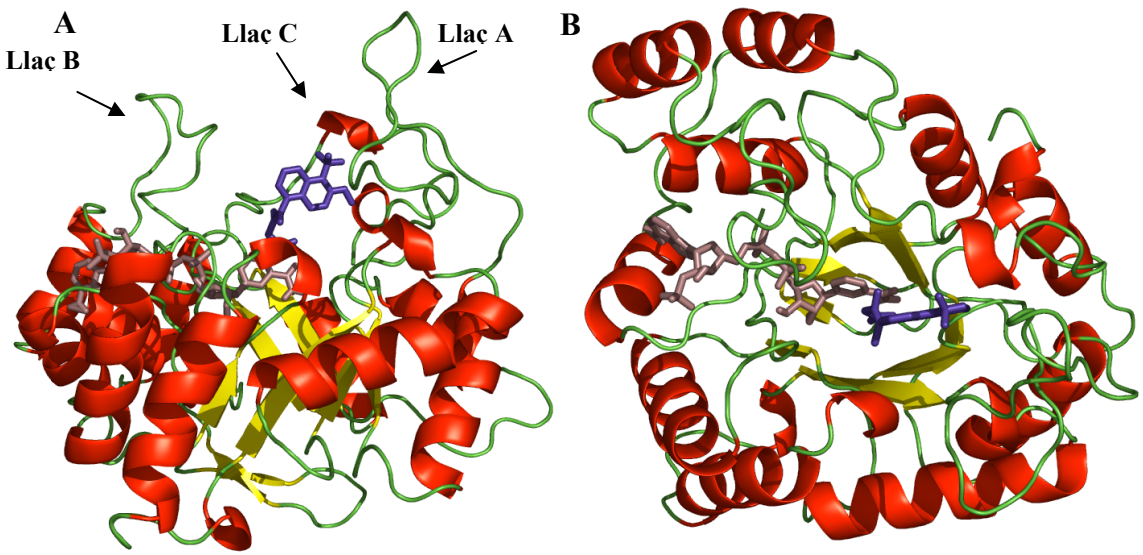


**Fig. 1. Estructutra tridimensional de l'aldosa reductasa unida a $NADP^+$ i tolrestat.** En A, s'indiquen els tres llaços d'unió al substrat. En B, es mostra la cara superior del barril, obtinguda al girar 90º la vista de A. El tolrestat està representat en lila i el $NADP^+$ en rosat (PDB 1AH3 extret de Urzhumtsev *et al.,* 1997). La figura va ser creada amb el programa PYMOL (http://www.pymol.org).

Les AKR comprenen unes 120 proteïnes, distribuïdes en 15 famílies enzimàtiques (AKR1-AKR15), que han estat agrupades segons criteris filogenètics (Jez *et al.,* 1997). Sense dubte, la més estudiada i nombrosa és la família AKR1, on hi podem trobar les subfamílies de l'aldehid reductasa (AKR1A), l'aldosa reductasa (AR; AKR1B), la hidroxiesteroide deshidrogenasa (AKR1C) o la cetoesteroide reductasa (AKR1D). Tot i que la majoria d'AKR són monòmers, les famílies AKR2, AKR6, i AKR7 poden formar multímers (Hyndman *et al.,* 2003). A la Taula 1, es recullen els enzims descrits en humans per a cada subgrup enzimàtic de la família AKR1, la família enzimàtica en la qual es centra aquesta Tesi.

Taula 1. **Enzims humans de la família AKR1**

| SUBFAMÍLIA | ENZIMS HUMANS |
|---|---|
| AKR1A | AKR1A1 |
| AKR1B | AKR1B1 |
| | AKR1B10 |
| AKR1C | AKR1C1 |
| | AKR1C2 |
| | AKR1C3 |
| | AKR1C4 |
| AKR1D | AKR1D1 |

1.2 Aldehid reductasa

L'aldehid reductasa (AKR1A) és una subfamília enzimàtica amb un tret estructural molt característic. Aquest enzim presenta una inserció de 9 residus en el llaç C, que determina una especificitat de substrat i inhibidor pròpia (Barski *et al.,* 1996). Per exemple, en un estudi del nostre grup (Crosas *et al.,* 2003) l'homòleg porcí d'aquesta subfamília (AKR1A2) es va mostrar inactiu enfront de la glucosa i el tot-*trans*-retinal. Per aquest motiu, hem exclòs aquesta subfamília del present estudi.

1.3 Aldosa reductasa

L'aldosa reductasa (AKR1B) és una subfamília enzimàtica present en diverses espècies de mamífers, com la humana, la rata, el ratolí, o el porc entre altres. El seus membres són capaços de reduir un elevat nombre d'aldehids diferents, incloent-hi catecolamines, esteroides, isocorticosteroides, isocaproaldehid, i aldehids derivats de la peroxidació lipídica. Com s'explica més endavant, aquesta subfamília està formada per dos subgrups enzimàtics diferents: l'anomenat pròpiament aldosa reductasa, i l'aldosa reductasa-*like*. En aquest apartat, ens referirem només al primer, doncs és el més estudiat i representatiu de la subfamília AKR1B.

La funció fisiològica d'aquests enzims és encara una incògnita, però la variant humana (AKR1B1) ha estat implicada en el desenvolupament de les complicacions secundàries de la

diabetis. Juntament amb la sorbitol deshidrogenasa (SDH), l'AKR1B1 forma part de la "via del poliol" (Figura 2). Amb aquest nom es coneix el procés en que es genera fructosa a partir de glucosa. AKR1B1 és la responsable de l'etapa limitant d'aquesta ruta metabòlica, on l'enzim genera sorbitol, un component altament osmòtic, a partir de glucosa.

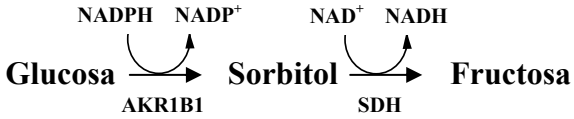


Figura 2. **Esquema de la via del poliol**

La participació d'AKR1B1 en la via del poliol és acceptada en el ronyó i els testicles, així com en els diferents teixits i òrgans en condicions d'hiperglicèmia. Però, degut a les constants cinètiques que presenta l'enzim per a la reducció de la glucosa ($K_m$ = 76 mM i $k_{cat}$ = 97 $min^{-1}$, Crosas *et al.,* 2003), es creu que en condicions normoglicèmiques el flux per aquesta via és molt limitat. A més, l'expressió d'aquest enzim en molts altres teixits, com el cor, vasos sanguinis, múscul esquelètic, cervell, i sobretot, glàndula adrenal, fa pensar que pot estar implicat en altres processos. L'elevat caràcter hidrofòbic del seu centre actiu no sembla destinat a la reducció de sucres, i és per això que molts autors han intentat aclarir el paper que l'enzim podria tenir en altres rutes metabòliques. Per exemple, s'ha vist que AKR1B1 mostra preferència per substrats de marcat caràcter hidrofòbic, com esteroides o aldehids derivats de lípids, en especial el 4-hidroxi-2-nonenal (Petrash 2004). Finalment, diferents estudis han demostrat que AKR1B1 participa en cascades de senyalització cel·lular mitjançant l'activació de PKC. El mecanisme pel qual actua és encara desconegut, però es postula que podria ser a través del producte d'una reacció catalitzada per l'enzim. S'han publicat diferents treballs en què es proposen altres possibles funcions de l'enzim, sobretot lligades al control de la proliferació cel·lular (Ramana *et al.,* 2002; Ramana *et al.,* 2003; Varma *et al.,* 2003).

### 1.4 Aldosa reductasa-*like*

Aquest és un grup d'enzims que, tot i formar part de la subfamília de l'aldosa reductasa (AKR1B), presenta unes característiques que el distingeixen. No va ser fins els anys 90 que es van descriure els seus primers membres. Inclou enzims amb una identitat seqüencial del 80-90%, sobretot descrits en rosegadors. Així, en ratolí s'han caracteritzat la *mouse vas deferens protein*

(MVDP o AKR1B7, Pailhoux *et al.,* 1990) i la *fibroblast-growth factor 1-regulated protein* (FR-1 o AKR1B8, Donohue *et al.,* 1994). En la línia cel·lular CHO, s'ha caracteritzat la CHO reductasa (AKR1B9, Hyndman *et al.,* 1997). En humans, s'ha descrit la *human small intestine*-reductase (HSI-reductase o aldosa reductasa-*like* o AKR1B10, Cao *et al.,* 1998; Hyndman and Flynn 1998).

El criteri principal per assignar un enzim al grup de l'AR-*like* és la identitat seqüencial. Entre la resta de membres de la subfamília de l'AR i el grup de l'AR-*like,* aquest valor és d'un 70%, inferior a la identitat entre membres del grup. També podem destacar-ne altres propietats diferenciadores, com la nul·la capacitat per reduir glucosa (Crosas *et al.,* 2003), o la seva distribució en teixits. Per exemple, en el cas de l'enzim humà, AKR1B10 no té una presència tan ubiqua com AKR1B1, però en canvi, mostra uns nivells superiors en còlon, intestí prim, i fetge (Cao *et al.,* 1998). Tot això fa pensar que es tracta de grups d'enzims amb funcions diferents. Una altra diferència amb la resta de la subfamília de l'AR és que hi pot haver més d'un representant per espècie. Ja hem vist el cas del ratolí, però també en rata es coneixen quatre aldosa reductases-*like* diferents (Zeindl-Eberhart *et al.,* 2001). Aquest fet planteja una possible multiplicitat de formes, algunes d'elles encara per descobrir.

Des que es va descriure AKR1B10 per primera vegada, l'enzim sempre ha estat associat a tumors de diferents tipus, principalment hepatocarcinoma cel·lular humà (HCC). Així, Cao i col. van observar que el 54% dels HCC analitzats presentava nivells elevats de mRNA per a AKR1B10 (Cao *et al.,* 1998). Altres autors han trobat resultats similars (Scuric *et al.,* 1998; Zeindl-Eberhart *et al.,* 2004). Recentment, també s'ha descrit que els nivells d'AKR1B10 estan augmentats en carcinoma de pulmó de cél·lules no petites (NSCLC), sobretot en els casos lligats a consum de tabac, fins i tot en estadis precancerosos (Fukumoto *et al.,* 2005). En concret, l'enzim es trobava sobrexpressat en el 84% dels casos de carcinoma de cèl·lules escamoses, i en el 29% d'adenocarcinomes. Aquest fet ha suggerit una possible participació de l'enzim en el procés de carcinogènesi de NSCLC. Recentment, s'ha proposat que l'augment de l'activitat retinal reductasa d'AKR1B10 podria afavorir l'aparició de NSCLC (Penning 2005).

### 1.5 Inhibidors de l'aldosa reductasa

Sense dubte, el paper d'AKR1B1 en situacions patològiques (tant en diabetis com en arteriosclerosi, entre altres) és el que ha despertat més interès. És clar, i així s'ha demostrat en models animals (Lee *et al.,* 1995), que AKR1B1 juga un paper importantíssim en aquests casos. Per això, s'han desenvolupat diferents inhibidors específics de l'enzim (ARI), amb la finalitat de

poder-los utilitzar en el tractament d'aquestes patologies. Clàssicament, s'han definit dos grans grups d'ARI:

- Les imides cícliques, com és el fidarestat:

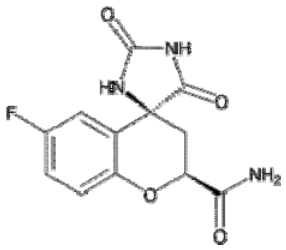


Figura 3. **Estructura del fidarestat**

- Els derivats d'àcid carboxílic, com el tolrestat:

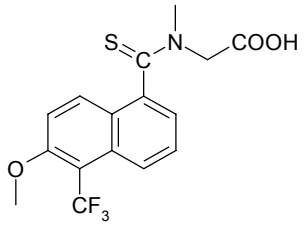


Figura 4. **Estructura del tolrestat**

Cal dir que, actualment, es fan molts esforços en el desenvolupament de nous inhibidors. Fins al moment, però, cap dels utilitzats ha tingut bons resultats clínics, tot i que alguns, com el tolrestat, van ser comercialitzats durant algun temps (només el fidarestat és comercialitzat actualment al Japó). Principalment, els problemes són deguts a la toxicitat i l'acció inespecífica que mostren *in vivo* (Jez *et al.,* 1997). En el cas del tolrestat, quan es van fer els assajos clínics es va decidir descartar el seu ús degut a l'hepatotoxicitat que presentava (Foppiano and Lombardo 1997). De totes formes, l'ús del tolrestat com a representant clàssic dels ARI en l'estudi d'AKR1B1 segueix sent habitual. Sovint, l'especificitat d'un ARI *in vitro* es determina comparant la seva capacitat d'inhibició front a AKR1B1 i AKR1A1. El nostre grup ha estudiat l'efecte dels ARI sobre AKR1B10 (Crosas *et al.,* 2003).

### 1.6 Estudis previs sobre l'estructura d'AKR

Fins a l'actualitat, mai no s'ha obtingut cap estructura cristal·lina d'un enzim del subgrup de l'aldosa reductasa unit a un substrat o anàleg d'aquest. De fet, són molt pocs els casos d'AKR cristal·litzades formant un complex amb un dels seus substrats. Els estudis realitzats es basen en la utilització de substrats per als que l'enzim estudiat presenta valors de $k_{cat}$ extremadament

baixos (és el cas del complex AKR1C2-testosterona, PDB 1J96, on l'enzim mostra un valor de $k_{cat}$ = 0,0071 $min^{-1}$, Nahoum *et al.,* 2001), el que facilita l'estabilització del complex format entre proteïna i substrat. En canvi, són nombroses les estructures obtingudes de complexos AKR-inhibidor. Així, la recerca de nous ARI sempre ha anat lligada a l'estudi estructural d'AKR. És també el cas del fidarestat, del què se n'ha obtingut l'estructura cristal·lina de diferents complexos proteics d'AKR1B1 (PDB 1EF3 i 1PWM), del mutant d'AKR1B1 Leu301Pro (PDB 2AGT), així com d'AKR1A2 (PDB 2AO0). Aquests treballs han demostrat que el pont d'hidrogen establert entre el grup amino de Leu301 d'AKR1B1 i el grup amida exocíclic del fidarestat és clau per a una més elevada especificitat de l'inhibidor per AKR1B1 que per AKR1A1 (Pro301) (Petrova *et al*., 2005). En el cas de l'inhibidor tolrestat, també s'ha realitzat un estudi profund per intentar establir els patrons estructurals determinants de la seva especificitat. Així, s'ha obtingut l'estructura cristal·lina per als complexos proteics de l'inhibidor amb AKR1B6 (l'homòleg porcí d'AKR1B1) (PDB 1AH3) i AKR1A2 (PDB 1AE4). A partir d'aquestes estructures, i juntament amb treballs de mutagènesi dirigida (Barski *et al*., 1996), s'ha observat que els impediments estèrics entre Arg312 d'AKR1A2 i l'inhibidor son els principals responsables per al menor efecte del tolrestat sobre AKR1A1 (El-Kabbani *et al*., 1997). Fins el present treball, no s'havia ressolt l'estructura d'AKR1B10, de manera que no es podien dur a terme estudis sobre els determinants estructurals per a la seva unió a substrats i inhibidors, i que expliquessin la seva especificitat per retinoides.

### 1.7 Hidroxiesteroide deshidrogenases

Aquest grup d'enzims es caracteritza per presentar activitat 3α-hidroxiesteroide deshidrogenasa. En humans, s'han descrit quatre isoenzims que comparteixen un 86% d'identitat seqüencial: AKR1C1 (o 20α(3α)-HSD); AKR1C2 (o 3α-HSD tipus 3); AKR1C3 (o 3α-HSD tipus 2) i AKR1C4 (o 3α-HSD tipus 1). Tots quatre presenten activitat 3-, 17-, i 20-cetoesteroide reductasa i 3α-, 17β- i 20α-hidroxiesteroide deshidrogenasa *in vitro*. Però estudis amb transfeccions transitòries en cèl·lules COS-1 van demostrar que, degut a la seva preferència pel NADP(H), només actuaven com a cetoesteroide reductases. S'ha proposat que les AKR1C podrien participar en la regulació dels nivells d'ocupació dels receptors d'andrògens, estrògens i progesterona (Penning *et al.,* 2004). Recentment s'ha demostrat la relació de les AKR1C amb els retinoides. Així, es va provar que la AKR1C7 bovina presentava activitat retinal reductasa (Endo *et al.,* 2001). A més, l'expressió d'un dels enzims humans, la AKR1C3, és induïda per l'àcid retinoic (Mills *et al.,* 1998).

## 2 Les alcohol deshidrogenasases de vertebrats

En vertebrats, l'alcohol deshidrogenasa (ADH) és un enzim citosòlic que pertany a la superfamília de les MDR i que es localitza de forma abundant en fetge, on pot representar el 3 % de la proteïna soluble (Edenberg i Bosron, 1997). L'enzim es detecta també en la resta de teixits de forma més o menys extensa, i presenta activitat davant una àmplia varietat d'alcohols i aldehids.

### 2.1 Propietats estructurals

L'ADH està composada per dues subunitats de 350 – 400 residus d'aminoàcids, que formen un dímer de pes molecular aproximat de 80.000 dalton. Cada subunitat presenta un domini de fixació del coenzim i un domini catalític, separats per una escletxa que constitueix el centre actiu. Aquesta escletxa té una longitud de 15 – 20 Å, equivalent a la llargada d'una cadena alifàtica de 9 – 10 carbonis (Eklund i Brändén, 1987), i queda accessible gràcies al petit canvi conformacional que es produeix després de la unió del coenzim. En el dímer, els dos dominis de fixació del coenzim es situen en el nucli de la molècula, formant una regió d'interfase entre les dues subunitats constituïda per 12 plegaments en làmina β (Brändén *et al*., 1975) (Figura 5). Variacions en aquests residus, i en els residus que constitueixen el lloc d'unió del coenzim, són responsables de les diferents propietats cinètiques dins les ADH. Així, l'especificitat per al coenzim $NAD^+$ o $NADP^+$, està determinada en part pel residu 223, Asp per als enzims dependents de $NAD^+$, o Gly o altres residus no acídics per als enzims dependents de NADP (Peralba *et al*., 1999). En un treball recent, el nostre grup va demostrar que la introducció de les tres mutacions simples Gly223Asp/Thr224Ile/His225Asn invertia totalment l'especificitat de coenzim de l'ADH8 de *Rana perezi* (Rosell *et al.,* 2003). Els dos dominis catalítics constitueixen la part externa del dímer, i contenen dos àtoms de zinc cadascun. Un d'aquests àtoms es situa a l'interior del domini catalític, i participa en la catàlisi. L'altre es situa en una regió superficial, i és essencial en el manteniment de l'estructura i l'estabilitat de l'enzim (Figura 5) (Brändén *et al*., 1975).

2.2 La família enzimàtica

En mamífers, la família ADH és un sistema enzimàtic complex, composat per múltiples formes moleculars que s'han agrupat en cinc classes (ADH1 – ADH5), d'acord amb les seves característiques enzimàtiques i estructurals (Jörnvall and Höög 1995; Duester *et al.,* 1999) (Taula 2). Almenys set gens diferents codifiquen l'ADH humana. S'han identificat nou tipus diferents de subunitats ($\alpha$, $\beta_1$, $\beta_2$, $\beta_3$, $\gamma_1$, $\gamma_2$, $\pi$, $\chi$ i $\sigma$), que donen lloc a les diferents formes moleculars de l'ADH humana. Les subunitats s'uneixen dins de cada classe formant homodímers i, en el cas de la classe I, també heterodímers. Les classes detectades en humans també es troben en altres mamífers, els quals presenten, en general, menys complexitat en la classe I. El principal criteri de classificació de les ADH és la comparació de les diferents seqüències de proteïna. En aquest treball, ens centrarem en l'estudi de les ADH1 i ADH4 humanes.

Taula 2. **Classes d'alcohol deshidrogenasa en humans**

| Classe | Gen | Al·lel | Proteïna |
|---|---|---|---|
| **I** | *ADH1A* | | ADH1A |
| | *ADH1B* | *ADH1B*1* | ADH1B1 |
| | | *ADH1B*2* | ADH1B2 |
| | | *ADH1B*3* | ADH1B3 |
| | *ADH1C* | *ADH1C*1* | ADH1C1 |
| | | *ADH1C*2* | ADH1C2 |
| **II** | *ADH2* | | ADH2 |
| **III** | *ADH3* | | ADH3 |
| **IV** | *ADH4* | | ADH4 |
| **V** | *ADH5* | | – [a] |

[a] No s'ha detectat.

2.3 *ADH1*

ADH1 és l'ADH clàssica, molt activa amb etanol, present en el fetge de tots els vertebrats, i molt sensible a la inhibició per 4-metilpirazole ($K_i$ < 2 μM). A part del fetge, també s'ha localitzat en altres òrgans com ronyó, pulmó i intestí, entre d'altres (Julià *et al.,* 1987; Boleda *et al.,* 1989). L'enzim constitueix la major activitat per metabolitzar l'etanol ingerit. Els valors de

$K_m$ per a l'oxidació de l'etanol varien per als diferents isoenzims d'ADH1, però en general són més baixos que per a les altres classes d'ADH. L'isoenzim que presenta una $K_m$ més baixa per a l'etanol (50 μM, pH 7,5) és el dímer de l'ADH1B1 ($\beta_1\beta_1$), mentre que l'isoenzim format pel dímer de l'ADH1B2 ($\beta_2\beta_2$), és el que presenta una major $k_{cat}$ (800 $min^{-1}$, pH 7,5).

A part de l'activitat amb l'etanol, l'ADH1 presenta una àmplia especificitat de substrat, i és activa amb alcohols primaris i secundaris, alcohols aromàtics i diols (Pietruszko, 1979). D'altra banda, l'enzim presenta una $K_m$ més baixa per als alcohols de cadena llarga que per als de cadena curta, mentre que la $k_{cat}$ és manté constant. Això fa que l'eficiència catalítica ($k_{cat}/K_m$) sigui més alta per a alcohols de cadena llarga. Entre els substrats fisiològics de l'ADH1 trobem ω-hidroxiàcids grassos (Björkhem 1972; Boleda *et al.,* 1993), aldehids formats en la peroxidació lipídica com el 4-hidroxi-2-nonenal (Boleda *et al.,* 1993), 3β-hidroxi-5β-esteroides (McEvily *et al.,* 1988), alguns metabolits de la dopamina, noradrenalina i serotonina (Mårdh and Vallee 1986; Svensson *et al.,* 1999) i retinoides (Boleda *et al.,* 1993; Yang *et al.,* 1994). El paper fisiològic de l'ADH1 en el metabolisme de l'etanol i dels retinoides s'ha demostrat gràcies a l'estudi de ratolins transgènics *Adh1-/-* que, encara que viables, mostren una major sensibilitat a la intoxicació per etanol i retinol (Deltour *et al.,* 1999b; Molotkov *et al.,* 2002a; Molotkov *et al.,* 2002b).

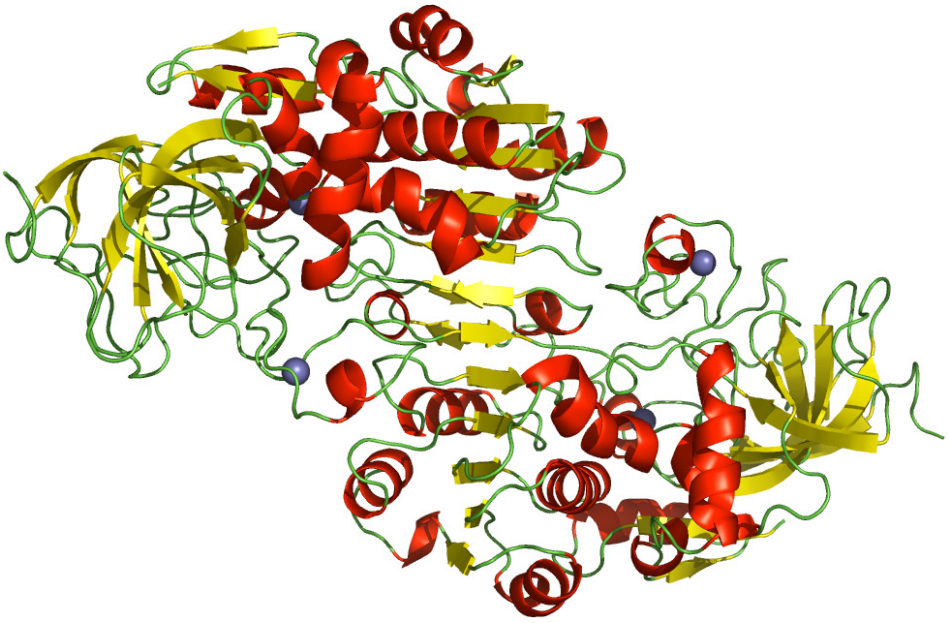

Figura 5. **Estructura de l'alcohol deshidrogenasa.** Es presenta l'estructura de l'ADH8 de *Rana perezi* (PDB 1P0C). L'estructuració de la cadena d'aminoàcids en hèlix α s'indica de color vermell i en làmina β de color groc. També es mostren els àtoms de zinc (lila). La figura va ser creada amb el programa PYMOL (http://www.pymol.org).

### 2.4 *ADH4*

L'ADH4 es troba distribuïda en diferents mucoses, teixits epitelials i òrgans externs, com la mucosa bucal, esòfag, tracte gastrointestinal, ull, pell i òrgans sexuals, però no en fetge (Moreno and Pares 1991). L'enzim presenta activitat amb etanol i amb compostos aromàtics, però té una major eficiència catalítica amb substrats de cadena lineal mitjana i llarga. A pH 7,5, la $K_m$ de l'ADH4 per a tots els substrats alifàtics és més alta que la de l'ADH1 i l'ADH2, però els valors de $k_{cat}$ de l'ADH4 també són els més alts d'entre totes les ADH humanes. L'ADH4 mostra una sensibilitat a la inhibició per 4-metilpirazole inferior a l'ADH1.

L'ADH4 és especialment activa amb aldehids produïts en la peroxidació lipídica (*trans*-2-hexenal i 4-hidroxi-2-nonenal) i ω-hidroxiàcids grassos (Boleda *et al.,* 1993; Allali-Hassani *et al.,* 1998). Si es té en compte la seva distribució i especificitat de substrat, es suggereix que l'ADH4 podria tenir un paper protector com a primera tanca metabòlica davant alcohols i aldehids tòxics.

L'ADH4 també és molt activa amb els retinoides (Boleda *et al.,* 1993; Yang *et al.,* 1994; Allali-Hassani *et al.,* 1998), i s'ha proposat que pot tenir una funció en la síntesi de l'àcid retinoic, durant el desenvolupament i en el manteniment i protecció dels teixits epitelials.

D'altra banda, l'alta activitat amb l'etanol i la presència de l'ADH4 en estómac han fet que es proposés la implicació de l'enzim en el metabolisme de primer pas de l'etanol ingerit en humans (*first – pass metabolism*) (Yin *et al.,* 1990; Moreno and Pares 1991; Seitz *et al.,* 1993).

## 3 Les deshidrogenases / reductases de cadena curta

Les deshidrogenases/reductases de cadena curta (SDR) presenten subunitats d'aproximadament 250 residus, amb un pes molecular de 25–30 kDa, i estan caracteritzades per un centre actiu amb el motiu YXXXK, sense ió metàl·lic en la seva estructura, a diferència de les ADH (Jörnvall *et al.,* 1995). Com en el cas de les ADH, però, les SDR presenten un domini de plegament de Rossmann per a la unió del cofactor (Figura 6).

Aquests enzims presenten activitat amb molts alcohols i aldehids fisiològics, entre els què destaquen els hidroxiesteroides i també els retinoides (Duester 2000). Es poden trobar en citoplasma, mitocondria, nucli, peroxisomes i reticle endoplasmàtic (Belyaeva, *et al.*, 2003). Segons les diferents famílies, utilitzen NAD(H) o bé NADP(H) com a coenzim.

L'activitat d'aquests enzims amb retinoides ha fet sorgir un debat sobre la participació relativa de cada família enzimàtica en el metabolisme de la vitamina A. Per això, a continuació s'amplia la descripció de les SDR humanes actives amb retinoides, informació que serà important en el desenvolupament de la discussió d'aquest treball.

### 3.1 Retinol deshidrogenases de la superfamília SDR

No existeix una classificació ni una nomenclatura ordenada per a les SDR actives amb retinoides. Aquesta família enzimàtica presenta una distribució subcel·lular caracteritzada per l'associació a membrana del reticle endoplasmàtic. Depenent de l'enzim, el centre catalític d'aquest pot trobar-se orientat cap el costat citosòlic de la membrana (és l'exemple de la RDH11 humana, Belyaeva, *et al*., 2003) o ancorat de manera que el centre catalític es posicioni orientat cap el lumen (com es va observar per a la RDH5 bovina, Simon, *et* al., 1999). En aquest treball, només tindrem en compte aquells enzims humans que presenten una distribució àmplia, descartant totes aquelles SDR humanes actives amb retinoides l'expressió de les quals es limita al teixit ocular.

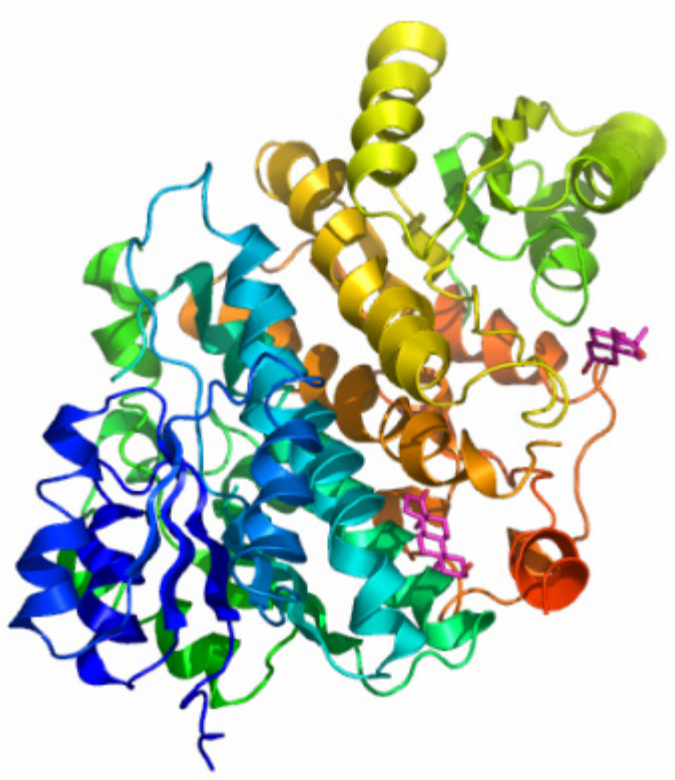

Figura 6**. Estructura tridimensional de la retSDR2 humana unida a etiocolanolona.**
Imatge extreta del PDB 1YB1. En color lil·la es representa el lligand.

### 3.2 *RoDH*

S'han descrit les retinol deshidrogenases RoDH1, RoDH2 i RoDH3, que s'han localitzat només en rosegadors (Chai *et al.,* 1995a; Chai *et al.,* 1995b; Chai *et al.,* 1996). En humans, s'ha descrit la RoDH4, enzim microsomal que a diferència de les RoDH de rosegadors que tots ells utilitzen NADP(H), utilitza preferentment NAD(H) (Gough *et al.,* 1998), encara que presenta similar especificitat de substrat, amb més activitat per als compostos tot-*trans* que per als isòmers *cis*. La RoDH4 s'ha detectat en fetge adult i fetal, i en pulmó fetal.

### 3.3 *RDH5*

Aquesta retinol deshidrogenasa s'ha detectat en ratolí, bou i en humans. L'enzim es localitza en l'epiteli pigmentat de la retina (Driessen *et al.,* 1995; Simon *et al.,* 1995), però també en altres teixits, com fetge, ronyó i cervell (Romert *et al.,* 1998; Gamble *et al.,* 1999). És un enzim microsomal que utilitza NAD(H) i que presenta activitat amb els derivats 11-*cis*- i 9-*cis*-del retinol i del retinal (Gamble *et al.,* 1999), però no amb el tot-*trans*. La funció fisiològica més important de la RDH5 és intervenir en la regeneració del pigment visual 11-*cis*-retinal, per oxidació dependent de NAD del 11-*cis*-retinol. Mutacions puntuals en el gen que codifica la RDH5 retarden l'adaptació a la foscor i causen *fundus albipunctatus*, que es relacionarien amb un metabolisme deficient del 11-*cis*-retinol (Yamamoto *et al.,* 1999). Estudis amb ratolins *knock-out* per a la RDH5 no han confirmat la importància de la proteïna en el cicle visual. Els ratolins tenen una visió normal, encara que presenten una acumulació superior a la normal d'èsters de *cis*-retinol a la retina (Driessen *et al.,* 2000; Shang *et al.,* 2002).

### 3.4 RoDH-*like* 3α-HSD

La primera caracterització de l'enzim va mostrar que aquest exhibia una clara preferència pel NAD(H) i activitat 3α-hidroxiesteroide deshydrogenasa, oxidant el 3α-androstadiol a l'androgen dihidrotestosterona (Biswas and Russell 1997). Aquesta activitat, juntament amb la seva elevada expressió en pròstata i testicles, van fer pensar que la seva funció fisiològica era dur a terme aquesta reacció. Darrerament, però, s'ha vist que la RoDH-*like* 3α-HSD també presenta activitat tot-*trans*-retinol deshidrogenasa, no així amb altres isòmers del retinol (Chetyrkin *et al.,* 2001).

### 3.5 *RDH11*

La RDH11 humana, també coneguda amb el nom de RalR1 o PSDR1 (Kedishvili *et al.,* 2002), presenta un ampli patró d'expressió. La RDH11 ha estat de les poques SDR actives amb retinoides que han pogut ser purificades, el que ha permès determinar les seves constants catalítiques. La RDH11 mostra una clara preferència pel NADP(H), i una major eficiència catalítica per a la reducció de l'isòmer tot-*trans* del retinal (~150.000 $mM^{-1} \cdot min^{-1}$, Belyaeva *et al.,* 2003).

### 3.6 *RDH12*

La RDH12 es un nou membre de la superfamília de les SDR, que presenta un 79 % d'identitat seqüencial amb RDH11. S'expressa especialment en els fotoreceptors, però també en pàncrees i ronyó (Belyaeva *et al.,* 2005). Com la RDH11, aquest enzim ha pogut ser purificat, mostra preferència pel NADP(H), i presenta una major eficiència catalítica per a la reducció del tot-*trans*-retinal (~900.000 $mM^{-1} \cdot min^{-1}$). Mutacions en el gen que codifica RDH12 s'han associat amb certes formes de distròfia retiniana amb herència autosòmica recessiva (arRD, Janecke *et al.,* 2004). Recentment, un altre estudi ha revelat l'existència de 16 noves mutacions del gen relacionades amb el desenvolupament d'arRD, mutacions que causen una disminució de l'activitat retinal reductasa de l'enzim expressat transitòriament en cèl·lules COS-1 (Thompson *et al.,* 2005). RDH12 també reconeix com a substrats els productes de la peroxidació lipídica.

## 4 Proteïna cel·lular unidora de retinol

Dintre de les cèl·lules, tot-*trans*-retinol i retinal estan units majoritàriament a proteïnes cel·lulars d'unió a retinol (CRBP), de les quals, en humans se'n coneixen quatre tipus diferents (CRBPI-IV). D'aquestes, però, només la CRBPI presenta un patró d'expressió ampli, mentre que la resta només es poden trobar en determinats teixits (Belyaeva *et al.,* 2005). Les CRBP són proteïnes citosòliques, d'uns 15 kDa de pes molecular i que, en mamífers, estan altament conservades (Ong 1994). S'ha proposat que el paper fisiològic d'aquestes proteïnes és aportar retinol per a la síntesi d'àcid retinoic, però els treballs amb ratolins *knock-out crbp* -/- han demostrat que aquesta proteïna no és necessària perqué es doni aquest procés, però en canvi sí

per a un correcte emmagatzematge del retinol en forma d'èsters de retinol (Ghyselinck *et al.,* 1999)

## 5 Lecitina-retinol acil transferasa

La lecitina-retinol acil transferasa (LRAT) és un enzim de transmembrana d'uns 25 kDa de pes molecular, que forma èsters de retinol en diferents teixits (Ruiz *et al.,* 1999). En la majoria de tipus cel·lulars, els èsters de retinol són la forma d'emmagatzematge de la vitamina A. Aquests èsters són hidrolitzats quan hi ha necessitat de tot-*trans*-retinol, principalment per a la síntesi d'àcid retinoic. En l'epiteli pigmentat de retina, però, els ésters de retinol també són els substrats d'una isomerhidrolasa per a la producció del 11-*cis*-retinol, que alhora és oxidat a 11-*cis*-retinal, el cromòfor dels fotopigments dels fotoreceptors de la retina (Bok *et al.,* 2003).

La LRAT segueix un mecanisme seqüencial ordenat bi-bi, on l'enzim primer uneix i transfereix un grup acil de la posició sn-1 de la fosfatidilcolina al centre actiu, i després uneix el tot-*trans*-retinol i li transfereix el grup acil, generant l'èster de retinol (Shi *et al.,* 1993).

En estudis amb ratolins *knock-out lrat* -/-, es va observar clarament que la LRAT és l'únic enzim responsable de l'esterificació del retinol en fetge, pulmó, ronyó i ull (Batten *et al.,* 2004). En teixit adipós, en canvi, els nivells d'èsters de retinol van augmentar entre 2 i 3 vegades en els ratolins que no expressaven LRAT (O'Byrne *et al.,* 2005). Els autors del treball van proposar que aquests èsters de retinol es formen per una via dependent d'acil-CoA, que és coneix com activitat acil-CoA:retinol acil transferasa (ARAT). Aquesta altra ruta metabòlica, però, és minoritària en els ratolins salvatges.

## 6 Retinoides

El terme "retinoide" inclou un ampli ventall de compostos derivats de la vitamina A, ja siguin naturals o sintètics, amb o sense activitat biològica. En canvi, el terme "vitamina A" es refereix només a aquells compostos que presenten l'activitat biològica del retinol. Així, la vitamina A participa de forma essencial en una multitud de processos biològics, com el desenvolupament fetal, la regulació de la proliferació i diferenciació de molts tipus cel·lulars, o el cicle visual, on actua com a cromòfor.

## 6.1 Estructura química dels retinoides

El tot-*trans*-retinol, un alcohol primari de pes molecular 286, és el compost del qual s'originen els altres retinoides. L'estructura bàsica es divideix en tres dominis: en un extrem, un anell de ciclohexè o β-ionona i, en l'altre extrem, un grup polar que pot presentar diferents estats d'oxidació: alcohol, aldehid o àcid. Finalment, unint els dos extrems, hi ha una cadena alifàtica amb quatre dobles enllaços conjugats (Figura 7). Aquests dobles enllaços donen lloc a isòmers, essent els més freqüents els tot-*trans*, 9-*cis*, 11-*cis*, i 13-*cis*.

Els retinoides són compostos hidrofòbics molt inestables, que s'oxiden amb facilitat en presència d'oxigen, i que es degraden per efecte de la llum, que catalitza la isomerització dels dobles enllaços. Això obliga a manipular-los en atmosfera inerta, i sota llum vermella.

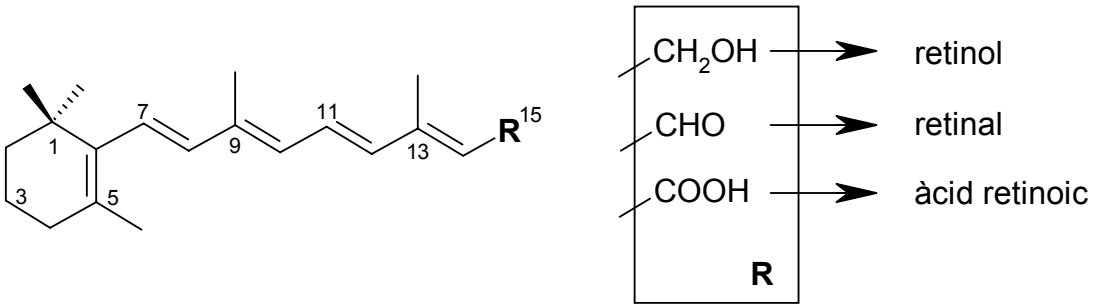


Figura 7. **Estructura química dels retinoides.** S'indica el nombre dels carbonis de la cadena principal, i la naturalesa de grup hidrofílic (R), que dóna lloc als diferents retinoides.

El Tween-80 (monooleat de sorbitan polioxietilènic), és un detergent no iònic i no desnaturalitzant. Durant molt temps, s'ha emprat per a la preparació de les solucions de retinoides amb l'objectiu de facilitar la solubilitat d'aquests, i augmentar la seva estabilitat. El seu ús ha estat molt ampli, tant en el nostre grup com en d'altres. La utilització d'aquest detergent, però, s'ha limitat als estudis amb enzims de les famílies de les ADH i AKR, no així amb SDR. Recentment, però, s'ha demostrat que la presència d'aquest compost causa una forta inhibició de tipus competitiu en les activitats de les ADH amb retinoides (Martras *et al.,* 2004).

### 6.2 Absorció, emmagatzematge, i transport de la vitamina A

La incorporació de vitamina A a l'organisme es produeix a través de la dieta: A partir de la ingesta de productes animals, la qual és una font d'èsters de retinol, i a partir de la ingesta de fruites i verdures, que són font, sobretot, de β-carotè.

En el primer cas, el retinol es absorbit pels enteròcits després que els èsters de retinol hagin estat hidrolitzats a l'intestí prim. En el segon cas, el β-carotè és absorbit directament per difusió passiva i, a l'interior dels enteròcits, és escindit en dues molècules de retinal gràcies a la 15,15'-dioxigenasa citosòlica. Posteriorment, el retinal es reduït a retinol (Figura 8).

A l'interior de l'enteròcit, el retinol és unit a la proteïna cel·lular unidora de retinol de tipus II (CRBP-II) i, per acció de la lecitina-retinol acil transferasa (LRAT), és esterificat amb àcids grassos de cadena llarga, per formar èsters de retinol. Aquests s'empaqueten en quilomicrons, i són transportats fins a les cèl·lules parenquimàtiques del fetge, on són captats mitjançant receptors específics. Allí, altre cop s'hidrolitzen per obtenir retinol que, unit a la proteïna plasmàtica unidora de retinol (RBP), és transferit a les cèl·lules estrellades del mateix òrgan. Aquestes cèl·lules emmagatzemen, en forma d'èsters de retinol, el 50-80% del retinol present a l'organisme.

Quan es requereix retinol en els teixits perifèrics, els èsters de retinol són hidrolitzats per l'enzim hidrolasa d'èsters de retinol (REH). El retinol generat es secretat i transportat per la sang en forma de complex retinol-RBP, que alhora és complexat amb la transtiretina per tal de disminuir-ne la filtració glomerular.

Més del 90% del retinol que entra a la cèl·lula es recicla cap al plasma, i només una petita part s'esterifica per ser emmagatzemat, s'activa a àcid retinoic, o bé és catabolitzat (Blomhoff 1994).

### 6.3 Mecanisme d'acció dels retinoides

Mentre que el retinol és la forma majoritària dels retinoides durant el seu transport i emmagatzematge, l'àcid retinoic representa la forma en què la vitamina A duu a terme la majoria de les seves funcions.

### 6.3.1 Síntesi de l'àcid retinoic

L'àcid retinoic es forma a partir de retinol a través de dues etapes d'oxidació. La primera comporta el pas de retinol a retinal, és una reacció reversible i l'etapa limitant del procés. La segona és l'oxidació de retinal a àcid retinoic (Figura 8).

Per a la conversió de retinol a retinal, s'han descrit membres de tres superfamílies enzimàtiques capaços de dur a terme la catàlisi: les alcohol deshidrogenases de cadena mitjana (ADH), les deshidrogenases/reductases de cadena curta (SDR) i les aldo-ceto reductases (AKR) (Figura 8). L'oxidació de retinal implica enzims de la família de les aldehid deshidrogenases (ALDH) i dels citocroms P450.

#### 6.3.1.1 Alcohol deshidrogenases

ADH1, ADH2 i ADH4 oxiden totes elles diferents isòmers de retinol *in vitro*, en presència de $NAD^+$ (Figura 8). De totes les ADH, l'enzim més actiu amb retinoides és l'ADH4. Durant el desenvolupament embrionari de ratolins, la colocalització de l'expressió dels gens de l'ADH1 i/o de l'ADH4 amb l'aparició d'àcid retinoic suggereix un paper actiu d'aquests enzims en la síntesi de l'àcid retinoic (Ang *et al.,* 1996; Haselbeck and Duester 1998). Aquesta colocalització també es dóna en epitelis que requereixen àcid retinoic per al seu funcionament, com l'epidermis (Haselbeck *et al.,* 1997), les mucoses de l'esòfag i l'estómac (Haselbeck and Duester 1997), i el tracte reproductiu masculí (Deltour *et al.,* 1997). D'altra banda, estudis genètics amb ratolins *knock-out* han demostrat la participació *in vivo* de l'ADH1, l'ADH4 i també l'ADH3 en el metabolisme de retinoides (Deltour *et al.,* 1999b; Molotkov *et al.,* 2002c; Molotkov *et al.,* 2004). Aquests estudis semblen demostrar que la funció de l'ADH1 en el metabolisme de retinoides és de protecció contra la toxicitat del retinol, mentre que l'ADH4 té un paper important en la síntesi de l'àcid retinoic durant el desenvolupament embrionari. En el cas de l'ADH4, a més de la colocalització de la proteïna i de la síntesi de l'àcid retinoic esmentada anteriorment, s'observa un descens de la supervivència fetal en estat de deficiència de vitamina A dels ratolins *knock-out* respecte als salvatges (Deltour *et al.,* 1999a; Molotkov *et al.,* 2002a). També s'ha relacionat l'ADH3, anomenada formaldehid deshidrogenasa dependent de glutatió, amb el metabolisme del retinol, perquè ratolins *knock-out* presenten un menor pes corporal, que es normalitza amb un suplement de vitamina A a la dieta (Molotkov *et al.,* 2002c). S'ha comprovat *in vitro* que l'activitat de l'ADH3 és 1400 vegades inferior a la de l'ADH1 i 4000 vegades inferior a la de l'ADH4 amb tot-*trans*-retinol, però la presència de l'ADH3 en tots els teixits, a diferència de la

resta d'ADH, li podria conferir un paper especial en el metabolisme dels retinoides (Molotkov *et al.,* 2002c). L'ADH2, malgrat que és activa amb retinol (Yang *et al.,* 1994; Svensson *et al.,* 1999), representa només un 2% de l'activitat retinol deshidrogenasa citosòlica del fetge. A més, presenta importants diferències cinètiques entre espècies, i per això es pensa que no deu tenir una funció conservada evolutivament com a retinol deshidrogenasa.

6.3.1.2 Deshidrogenases/reductases de cadena curta

Les retinol deshidrogenases/reductases de la superfamília SDR són enzims microsomals capaços d'oxidar retinol i de reduir retinal, utilitzant $NAD(P)^+$ i NAD(P)H com a coenzim, respectivament (Figura 8). Entre les primeres, s'han descrit diverses retinol deshidrogensases humanes (RoDH4, RoDH-*like* 3α-HSD, o RDH5) totes elles amb expressió extraocular. Entre les dependents de NADP(H), se n'han descrit algunes que només s'expressen en l'ull (retSDR1 i prRDH) i d'altres que s'expressen en un ventall més ampli de teixits (RDH11 i RDH12). Abans del present treball, es creia que les SDR podien tenir un paper més important que les ADH en el metabolisme de retinoides, ja que, aparentment, moltes d'elles utilitzen com a substrat el retinol unit a la proteïna CRBP (holo-CRBP), que és la forma majoritària en què es troba el retinol dins la cèl·lula. A més, els valors de $K_m$ determinats fins al moment per a SDR, eren dos ordres de magnitud inferiors que per a les ADH o AKR. Així, les SDR presentaven uns valors de $K_m$ per als retinoides molt més pròxims a les seves concentracions fisiològiques. Tot i això, existeixen dubtes sobre el paper real de les SDR *in vivo*, ja que les retinol deshidrogenases de cadena curta també presenten activitat amb esteroides, i sobretot, perquè en ratolins *knock-out crbpI* -/- s'observa una disminució de l'emmagatzematge de retinol en forma d'èsters de retinol, però no sembla alterat el seu metabolisme a àcid retinoic (Ghyselinck *et al.,* 1999).

6.3.1.3 Aldo-ceto reductases

L'activitat retinoide oxidoreductasa sempre s'havia adscrit a membres de les superfamílies MDR i SDR. Recentment, però, el nostre grup va caracteritzar en pollastre una nova AKR, AKR1B12, activa amb diferents isòmers de retinal i retinol (Crosas *et al.,* 2001). Aquest enzim mostra una identitat seqüencial del 66-69% amb dues AKR humanes que també s'han demostrat actives amb retinoides, AKR1B1 i AKR1B10 (Crosas *et al.,* 2003). Finalment, també s'ha descrit activitat retinal reductasa en la prostaglandina F sintasa 1 de bou (AKR1C7) (Endo *et al.,* 2001). Totes les AKR actives amb retinoides mostren una clara preferència per a la reducció de retinal.

La capacitat d'AKR1B10 per reduir retinal ha estat proposada com a un possible mecanisme que podria estar involucrat en el càncer de cèl·lules no petites de pulmó (NSCLC) (Penning, 2005). De totes formes, encara no s'ha realitzat cap estudi que demostri la implicació d'aquests enzims en el metabolisme dels retinoides *in vivo*.

#### 6.3.1.4 Oxidació de retinal

L'oxidació de retinal es pot donar per l'acció de membres de dues famílies enzimàtiques diferents: la família de les aldehid deshidrogenases (ALDH) i la família del citocrom P-450 (Figura 8). Existeixen diferents ALDH amb activitat retinal deshidrogenasa. A més, aquestes poden utilitzar tant el retinal lliure com el retinal unit a CRBP, essent RALDH2 l'enzim amb una major activitat catalítica (Duester, 2000). El citocrom P-4501A1 és el que mostra una major activitat dintre la seva família, però *in vitro* mostra uns valors de $K_m$ (12 mM amb retinal) de baix significat fisiològic.

### 6.3.2 Àcid retinoic

Un cop la cèl·lula ha sintetitzat l'àcid retinoic, aquest s'uneix a les proteïnes cel·lulars unidores d'àcid retinoic (CRABP) per ser transportat a l'interior del nucli. Existeixen dos tipus de receptors nuclears activables per àcid retinoic, els RAR i els RXR, ambdós amb els respectius subtipus $\alpha$, $\beta$ i $\gamma$. Tots dos pertanyen a la família de receptors d'hormones esteroïdals, tiroïdals, i de la vitamina D. Els RAR són capaços d'unir els isòmers tot-*trans*-retinoic i 9-*cis*-retinoic. Els RXR, en canvi, només uneixen 9-*cis*-retinoic (Figura 9). Un cop el receptor ha unit la molècula de retinoic, pot formar un dímer i així esdevenir actiu. Però mentre que els RAR només formen homodímers o heterodímers amb un altre receptor d'àcid retinoic RXR (RAR-RXR), els RXR també poden heterodimeritzar amb receptors d'hormona tiroïdal (RXR-TR), vitamina D (RXR-DR), i amb el receptor activat dels proliferadors de peroxisomes (RXR-PPAR). Un cop el receptor ha dimeritzat, pot unir-se a un element de resposta a l'àcid retinoic en el DNA (RARE o RXRE), per així activar o inhibir l'expressió d'un gen determinat.

En la ruta metabòlica de síntesi d'àcid retinoic a partir de retinol, la primera etapa, on s'oxida retinol a retinal de manera reversible, representa l'etapa limitant de velocitat del procés. Tradicionalment, els enzims descrits capaços de dur a terme aquesta reacció pertanyien a la família de les ADH, o bé a la de les SDR. El descobriment, per part del nostre grup (Crosas *et*

*al.,* 2001), que membres de les AKR també podien participar-hi, va obrir un ampli ventall d'incògnites en el camp.

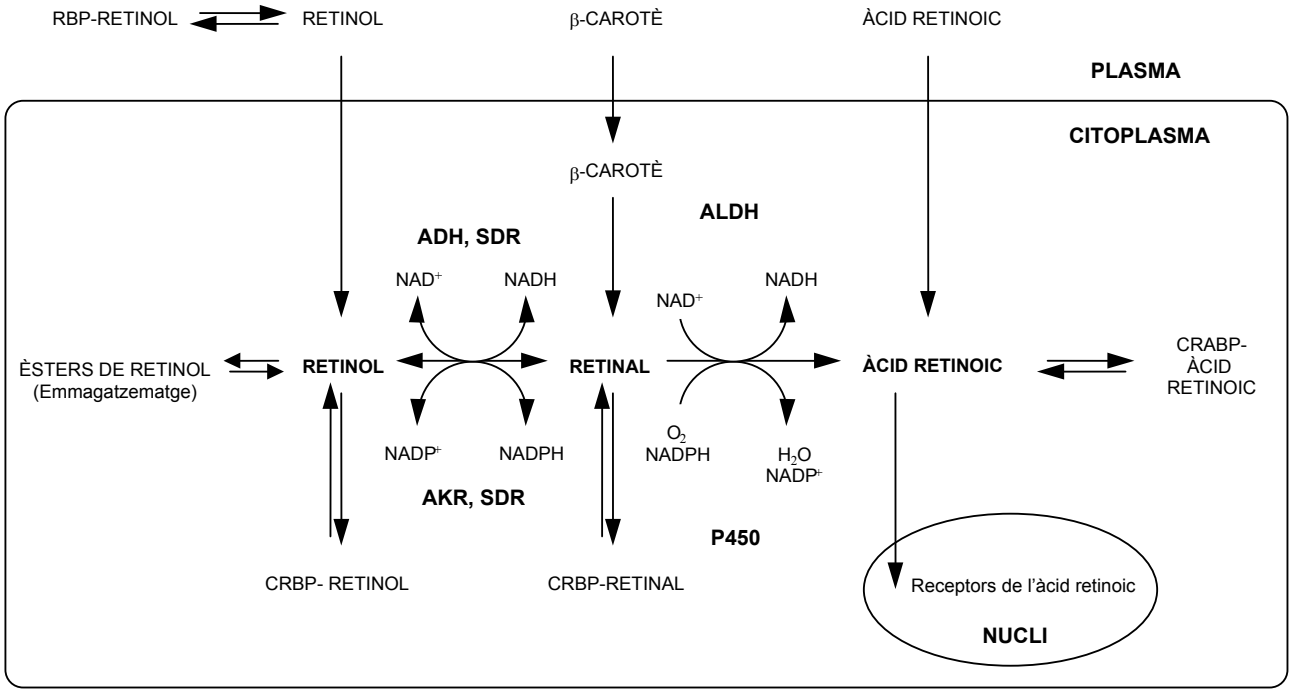


Figura 8. **Mecanisme proposat per a la síntesi de l'àcid retinoid i l'emmagatzemament de retinoides.** El retinol és transportat en el plasma unit a la proteïna RBP, abans d'entrar dins la cèl·lula. En el citoplasma, el retinol pot ser esterificat, o oxidat a àcid retinoic. Aquesta oxidació es produeix a través de dues reaccions consecutives d'oxidació, la primera de retinol a retinal, i la segona de retinal a àcid retinoic (Figura modificada de Duester, 1996).

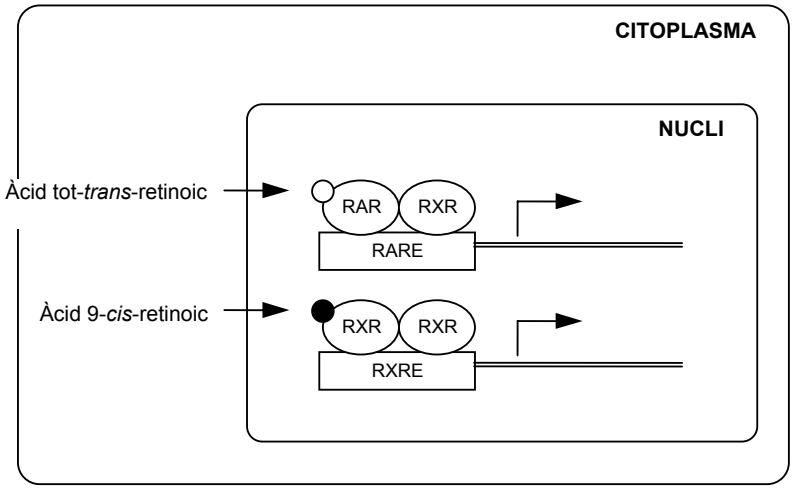


Figura 9. **Model del mecanisme d'acció dels retinoides.** Els àcids tot-*trans*- i 9-*cis*-retinoic interaccionen amb els corresponents receptors (RAR, RXR), formant dímers, abans d'unir-se als elements de resposta a àcid retinoic (RARE, RXRE) que desencadenaran la funció requerida.

# OBJECTIUS

**CAPÍTOL I:** Anàlisi comparativa de les activitats de MDR, SDR i AKR humanes amb retinoides lliures i units a CRBPI.

El paper relatiu dels enzims de cadascuna de les superfamílies enzimàtiques en el metabolisme de retinoides no s'havia pogut determinar per causa de les diferències metodològiques en els estudis cinétics anteriors. L'objectiu d'aquest capítol ha estat determinar les propietats cinètiques de membres de cada superfamília, en les mateixes condicions, per poder estimar la seva contribució al metabolisme de retinoides.

Objectius parcials:

**1.** Determinar l'efecte de la presència del detergent Tween-80 en l'activitat retinal reductasa de les AKR, emprant l'AKR1B1 com a model.

**2.** Determinar el valor de les constants cinètiques $K_m$ i $k_{cat}$ en l'oxidació del retinol i reducció del retinal, per a AKR, ADH i SDR humanes, utilitzant una metodologia basada en la solubilització de retinoides en BSA i anàlisi per HPLC.

**3.** Caracteritzar la capacitat d'enzims de cada superfamília per metabolitzar retinoides units a CRBPI.

**CAPÍTOL II:** Estudis estructurals i funcionals sobre l'AKR1B10

L'AKR1B10 és l'AKR humana més activa amb retinal i s'ha relacionat amb el desenvolupament de diferents formes de càncer. El segon objectiu de la Tesi ha estat aprofundir en la caracterització funcional de l'enzim i obtenir la seva estructura tridimensional.

Objectius parcials:

**1.** Determinar el valor de les constants cinètiques $K_m$ i $k_{cat}$ d'AKR1B10, en absència del detergent Tween-80, per a l'oxidació del retinol i reducció del retinal.

**2.** Estudiar el paper d'AKR1B10 en el metabolisme de retinoides en un entorn cel·lular, emprant cèl·lules COS-1 com a model.

**3.** Caracteritzar l'efecte de l'inhibidor tolrestat en l'activitat retinal reductasa d'AKR1B10 *in vitro* i *in vivo*.

**4.** Obtenir l'estructura cristal·lina de l'AKR1B10 unida a $NADP^+$ i tolrestat.

**5.** Caracteritzar la butxaca d'unió a tolrestat. Estudiar les diferències existents amb la butxaca d'unió a tolrestat d'AKR1B1.

**6.** Obtenir el model del complex AKR1B10-$NADP^+$-tot-*trans*-retinal. Estudiar els determinants estructurals que confereixen l'especificitat de substrat amb retinoides.

# CAPÍTOL I

## ANÀLISI COMPARATVA DE LES ACTIVITATS DE MDR, SDR I AKR HUMANES AMB RETINOIDES LLIURES I UNITS A CRBPI.

## INTRODUCTION

Retinoids participate in multiple cellular functions, mainly through the activation of nuclear receptors by retinoic acid binding (Mangelsdorf *et al.,* 1994). Depending on cellular needs, retinol obtained from circulation or produced locally from β-carotene (reviewed in Olson and Krinsky, 1995; Vogel, 1999; von Lintig and Wyss, 2001; Harrison, 2005) is either converted into the storage form, retinyl ester, by lecithin:retinol acyltransferase (LRAT) or directed toward retinoic acid biosynthesis (Blaner and Olson, 1994). This biosynthesis occurs in two steps: retinol is oxidized to retinaldehyde, and then retinaldehyde is oxidized to retinoic acid (reviewed by Napoli, 1999). The oxidation of retinol to retinaldehyde is a reversible reaction and the rate-limiting step of retinoic acid biosynthesis.

Cellular retinol binding proteins (CRBPs) are a group of cytosolic proteins that bind retinol and retinaldehyde with high affinity (reviewed by Noy, 2000). Four different types of CRBPs have been described (Ong and Chytil, 1975; Ong, 1984; Folli *et al.,* 2001; Vogel *et al.,* 2001), but only CRBPI has been shown to have a widespread distribution (Noy, 2000). CRBPI is a highly conserved protein that differs by five amino acid residues between rats and humans (Colantuoni *et al.,* 1985; Sundelin *et al.,* 1985). Its physiological function is not yet fully understood. It has been proposed that CRBPI-bound retinol serves as the prime substrate for LRAT and retinol dehydrogenases responsible for retinoic acid biosynthesis *in vivo* (Napoli, 1999).

Members of three oxidoreductase superfamilies have been implicated in human retinol metabolism. Historically, cytosolic ADH enzymes of the medium-chain dehydrogenase/reductase (MDR) family were the first enzymes found to be active toward retinoids (Zachman and Olson, 1961; Mezey and Holt, 1971). Among the human MDRs, ADH4 was shown to be the most catalytically efficient retinol dehydrogenase, followed by ADH2 and ADH1 (Yang *et al.,* 1994). Since most ADHs prefer $NAD^+$, the major oxidative cofactor in the cells (Veech *et al.,* 1969), these enzymes were proposed to function in the oxidative direction and to catalyze the conversion of retinol to retinaldehyde for retinoic acid biosynthesis. However, despite the high catalytic efficiency of ADHs, their role in retinoid metabolism has been questioned because of their high $K_m$ values for retinoids (Yang *et al.,* 1994) and their inability to recognize CRBPI-bound retinol as a substrate (Kedishvili *et al.,* 1998).

Short-chain dehydrogenases/reductases (SDRs) emerged as a novel type of all-*trans*-retinol dehydrogenases when it was reported that two of its members, rat microsomal RoDH-1 and RoDH-2, were capable of oxidizing free retinol as well as retinol bound to CRBPI (holoCRBPI) (Napoli *et al.,* 1992; Chai *et al.,* 1995a; Chai *et al.,* 1995b). In addition to retinoids, RoDH enzymes were found to oxidize the 3α-hydroxyl group on androgenic hydroxysteroids (Biswas and Russell, 1997). Three RoDH-related $NAD^+$-dependent SDR active toward all-*trans*-retinol have been characterized in humans (Gough *et al.,* 1998; Chetyrkin *et al.,* 2001; Soref *et al.,* 2001; Markova *et al.,* 2003). The reported $K_m$ values of these SDRs for retinoids were a ~100-fold lower than those of ADHs. The $k_{cat}$ values for the majority of microsomal RoDH-like SDRs had not been determined because of the difficulties with their purification. However, successful purification of a catalytically active human RoDH-4 was recently achieved by reconstitution of the purified enzyme into proteoliposomes (Lapshina *et al.,* 2003).

In addition to the $NAD^+$-dependent SDRs, several $NADP^+$-dependent microsomal SDRs with activities toward retinoids were identified. Some of these enzymes, mouse RDH11 and human RDH12, had a high activity toward short- and medium-chain aldehydes (Kasus-Jacobi *et al.,* 2003; Belyaeva *et al.,* 2005). Human RDH11 (Haeseleer *et al.,* 2002) also known as PSDR1 or RalR1, showed a widespread expression pattern and was proposed to catalyze the reduction of all-*trans*-retinaldehyde to all-*trans*-retinol in extraocular tissues (Belyaeva *et al.,* 2003; Kim *et al.,* 2005).

Most recently, the $NADP^+$-dependent aldo-keto reductases (AKRs) were defined as a new group of cytosolic enzymes that could contribute to the oxidoreductive conversions of retinoids. Members of the AKR1 family, such as chicken AKR1B12 (Crosas *et al.,* 2001), human aldose reductase (AKR1B1), human small intestine aldose reductase (AKR1B10) (Crosas *et al.,* 2003, and bovine prostaglandin F synthase (AKR1C7) (Endo *et al.*, 2001) were shown to preferentially reduce retinaldehyde to retinol with their $K_m$ values close to those of ADH isozymes but with lower $k_{cat}$ values. The activities of AKRs toward CRBPI-bound retinoids have not yet been examined.

Numerous studies were performed to clarify the relative importance of each enzyme group in retinol metabolism. However, never before the properties of the enzymes from the three superfamilies had been compared side-by-side using the same methodology. Recently, Tween-80, a detergent used in the assays of ADH activities to improve the solubility and stability of retinoids, was found to act as an apparent competitive inhibitor of human ADH4 and ADH1

(Martras *et al.,* 2004). This finding suggested that the $K_m$ values of ADHs for retinoids might have been overestimated.

Classically, SDR activities had been assayed differently than ADH and AKR activities. First of all, most studies on SDRs were carried out using microsomal preparations of the enzymes because purified SDRs were extremely unstable. Secondly, detergents strongly inhibited the activities of the membrane-bound SDRs (Kedishvili *et al*., unpublished observations), therefore, retinoids had to be solubilized by binding to bovine serum albumin. Finally, spectrophotometric product detection employed for ADHs and AKRs could not be used for SDRs because of the turbidity caused by the microsomal membranes, their intrinsic NADH oxidase activity, and the low sensitivity of the method. Hence, retinoid conversions by SDR enzymes were analyzed by high-performance liquid chromatography. The reaction conditions for ADHs, AKRs and SDRs, such as temperature or buffer composition, frequently varied from one group of enzymes to another, making the comparison of their properties difficult. Therefore, here for the first time, the activities of human enzymes from each superfamily were characterized using the same methodology in order to compare their catalytic efficiency for the oxidation of retinol and reduction of retinaldehyde, and to determine which enzymes can utilize CRBPI-bound retinoids.

## EXPERIMENTAL PROCEDURES

*Expression and Purification of AKR1B1 and AKR1C3.* The pBluescript SK+ vector (Stratagene, La Jolla, CA) containing human AKR1C3 cDNA (KIAA0119 in Nagase *et al.,* 1995) was kindly donated by Dr. T. Nagase of the Kazusa DNA Research Institute, Chiba, Japan. AKRs were expressed and purified as described by Crosas *et al*. (2003). Briefly, *E. coli* BL21 strain transformed with the plasmid pET16b, that encoded AKR1B1 fused to the C-terminal $His_{10}$ tag or with the plasmid pET-30 Xa/LIC that encoded AKR1C3 fused to the C-terminal $His_6$ tag, was grown in 2xYT medium at 24 ºC for 8 h. Protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Amersham Biosciences, Piscataway, NJ) to 1 mM. AKRs were purified using a nickel-charged chelating Sepharose® Fast Flow resin (Amersham Biosciences, Uppsala, Sweden). After washing with 60 mM imidazole in 20 mM Tris/HCl, 0.5 M NaCl, pH 7.9, the enzyme was eluted by a 0.06-1.0 M imidazole (Sigma-Aldrich, Saint-Louis, MO, USA) gradient in the same buffer. The absorbance of

fractions at 280 nm was analyzed using a Varian Cary 400 spectrophotometer. Fractions containing AKR were collected and dialyzed twice against 4 l of 10 mM sodium phosphate, 1 mM EDTA, pH 8.0.

*Expression and Purification of ADH1B2 and ADH4. E. coli* BL21 cells were transformed with the plasmid pGEX-4T-2 containing the cDNA for each enzyme as described by Martras *et al*. (2004). Liquid cultures in 2xYT medium were grown until saturation at 25 ºC. Zinc sulphate (10 μM) was added prior to induction with 0.1 mM IPTG, at 22 ºC, for 15 h. The vector pGEX-4T-2 allowed the ADH expression as a fusion protein with glutathione-S-transferase (GST). This ADH-GST protein construct was purified using the affinity resin glutathione-Sepharose 4B (Amersham Biosciences). After washing with 100 mM Tris/HCl, pH 7.0, 2.5 mM dithiothreitol (DTT, Sigma-Aldrich), 10 % glycerol, 0.2 M sodium chloride, 10 μM zinc sulphate, elution of the ADH was performed by thrombin digestion (10 U/mg protein, Amersham Biosciences) in the same buffer, for 15 h, at room temperature.

*Expression and Purification of Microsomal SDRs.* Sf9 insect cells (Invitrogen, Carlsbad, CA, USA) infected with recombinant baculovirus that contained each cDNA were grown for 3 days at 27 ºC. Cells were homogenized using a French pressure cell press and microsomes were isolated by differential centrifugation. Human RoDH-4 was purified as described previously (Lapshina *et al.,* 2003). Briefly, microsomal membranes containing RoDH-4 were solubilized using 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC) (Avanti Polar Lipids, Alabaster, AL, USA). DHPC extract was incubated with proteinase K at a 10:1 (w/w) ratio for 14 h on an ice bath. At the end of the incubation, proteinase K was inactivated by the addition of phenylmethylsulphonylfluoride. Proteinase K-treated extract was reconstituted into artificial lipid membranes in the presence of DHPC. The detergent was removed by dialysis against reconstitution buffer containing Biobeads SM2 beads (Bio-Rad, Hercules, CA, USA) at 4°C overnight. RoDH-4-containing vesicles were recovered and concentrated by flotation in a Nycodenz (Sigma-Aldrich) step gradient. The vesicles were stored for several months at 4°C without apparent loss of activity.

Human RDH11 was purified as described in Belyaeva *et al*. (2003). Briefly, Sf9 cells expressing RDH11-$His_6$ protein were homogenized and solubilized by the addition of DHPC. DHPC extract was supplemented with 5 mM imidazole and loaded onto a $Ni^{2+}$-NTA metal affinity column (Qiagen Inc., Valencia, CA, USA). After extensive washing, RDH11-$His_6$ was eluted with a gradient of 100-250 mM imidazole. To remove imidazole, the buffer in the eluate was exchanged for 100 mM potassium phosphate, pH 7.4, 40 mM potassium chloride, 1 mM

DHPC, 1 mM DTT, 20% glycerol, using PD-10 gel-filtration columns (Amersham Biosciences, Piscataway, NJ, USA). Purified RDH11-$His_6$ preparations were stored at -80°C for several months without loss of activity.

EST clone C152778 encoding RDH5 was obtained from Research Genetics (Huntington, AL). The full-length cDNA was amplified using primers 5'-GCT GGA TCC ATG TGG CTG CCT CTT CTG CT-3' (forward primer; *Bam*HI site underlined) and 5'-AGG GAA TTC TCA GTA GAC TGC TTG GGC AG-3' (reverse primer; *Eco*RI site underlined) and cloned into pVL1393 vector (PharMingen, San Diego, CA, USA). Preparation of the recombinant baculovirus, expression of the protein in Sf9 cells, and isolation of the microsomal fraction were carried out as described for RoDH-4 (Gough *et al.,* 1998).

*Expression of LRAT in Sf9 cells.* EST clone with the GenBank accession number BI461423, that contained the full-length cDNA for human LRAT (Ruiz *et al.,* 1999) (insert size ~2 kb) between the *BamH*I and *Sal*I/*Xho*I restriction sites of pBluescriptR vector, was obtained from American Type Culture Collection (Manassas, VA, USA) (catalog number MGC-33103). The coding region was amplified using primers that contained recognition sites for restriction endonucleases *Bgl*II and *Xba*I, respectively: forward, 5' - C TAC AGA TCT ATG AAG AAC CCC ATG CTG GAG - 3' (*Bgl*II site underlined); reverse, 5' - G GGT TCT AGA TTA GCC AGC CAT CCA TAG GAA G - 3' (*Xba*I site underlined). Amplification was carried out for 40 cycles with denaturing at 94°C for 1 min, annealing at 47°C for 1 min, and extension at 72°C for 1.5 min. The PCR product of 0.7 kb was purified by agarose gel electrophoresis and subcloned into pUC18 vector by blunt-end ligation. The cDNA for LRAT was excised from pUC18 vector using *Bgl*II and *Xba*I restriction endonucleases and cloned into the respective sites of the baculovirus transfer vector pVL1392 (PharMingen). The cDNA sequence was confirmed by sequencing. Recombinant baculovirus was prepared as described previously for RoDH-4 (Gough *et al.,* 1998). Expression and isolation of microsomes containing LRAT were done essentially as described above for microsomal SDRs.

*Preparation of apoCRBPI and holoCRBPI.* CRBP type I was expressed in *E. coli* either as an N-terminal fusion to glutathione S-transferase (GST-CRBPI) or as a C-terminal fusion to a bifunctional tag, consisting of the chitin binding domain (CBD) and the intein (CBD-intein). GST-CRBPI construct in pGEX-2T vector (Pharmacia, Piscataway, NJ, USA) was expressed in TG-1 *E. coli* cells and the fusion protein was purified to homogeneity using affinity chromatography on a glutathione-agarose column as described previously (Napoli *et al.,* 1992). CRBPI-CBD-intein fusion construct in pKYB1 vector (New England Biolabs Inc., Beverly, MA,

USA) was expressed in BL21(DE3) cells and purified using the IMPACT$^{TM}$-CN protein purification system (New England Biolabs Inc.) as described in Belyaeva *et al*. (2005). CRBPI was released from the intein tag after the induction of the cleavage reaction with 50 mM DTT.

To prepare holoCRBPI, an aliquot of purified apoCRBPI was saturated with a 2-fold molar excess of all-*trans*-retinol at room temperature for 1 h. Unbound retinol was removed by gel-filtration on a G50 Sephadex column (Lapshina *et al.,* 2003). The $A_{350}/A_{280}$ ratio of the holoCRBPI preparation was 1.76. HoloCRBPI was stored in small aliquots at –80° C.

*Protein Analysis.* Protein purity was analyzed by electrophoresis in SDS-polyacrylamide gel followed by staining with Coomassie® Brilliant Blue (Sigma-Aldrich). The protein concentration of AKR and ADH preparations was determined by Bradford (Bradford 1976). The protein concentration of microsomes was determined by Lowry (Lowry *et al.,* 1951).

*Enzyme Kinetics.* For ADHs and AKRs, standard activity assays were done before each experiment. Ethanol was used as a substrate for ADH1B2 and ADH4, and $_{D,L}$-glyceraldehyde for AKR1B1, as described previously (Crosas *et al.,* 2003; Martras *et al.,* 2004). AKR1C3 activity was measured with 1 mM 1-acenaphtenol and 2.3 mM NAD, in 100 mM potassium phosphate, pH 7.0, at 25 ºC (Penning *et al.,* 2000). The retinaldehyde reductase activity of AKR1B1 in the presence of Tween-80 was determined by following the absorbance at 400 nm, using a Varian Cary 400 spectrophotometer, in 0.1 M sodium phosphate, pH 7.5, at 25 ºC, with 0.2 mM NADPH (Sigma-Aldrich) in 0.2-cm path length cuvettes.

Assays of retinoid activities in the presence of bovine serum albumin (BSA) were performed in 90 mM potassium phosphate, pH 7.4, 40 mM KCl, at 37 ºC (reaction buffer), in siliconized glass tubes as described previously (Gough *et al.,* 1998). Stock solutions of retinoid substrates (Sigma) for AKRs and SDRs were prepared in ethanol. For LRAT, dimethyl sulfoxide (DMSO, Sigma) was used as a solvent (Gollapalli and Rando 2003). To avoid competitive inhibition effects, acetone was used for ADHs. Neither solvent exceeded 1% (v/v) in the reaction mixture. Working stock solutions of retinoids were prepared by a 10-min sonication in the reaction buffer in the presence of equimolar delipidated bovine serum albumin. The actual amount of solubilized retinoid was determined based on the corresponding extinction coefficients at the appropriate wavelength. The following molar absorption coefficients in aqueous solutions were used: $\varepsilon_{328}$ = 39500 $M^{-1}\cdot cm^{-1}$ for all-*trans*-retinol, $\varepsilon_{400}$ = 29500 $M^{-1}\cdot cm^{-1}$ for all-*trans*-retinaldehyde, $\varepsilon_{367}$ = 26700 $M^{-1}\cdot cm^{-1}$ for 9-*cis*-retinaldehyde. The reactions were started by the addition of cofactor and carried out for 5-30 min at 37 ºC. The following amounts of cofactors were used for each enzyme: 0.5 mM $NADP^+$ or NADPH for AKR1B1; 2.4 mM $NADP^+$ or 2.3

mM NADPH for AKR1C3; 2.4 mM $NAD^+$ or 1 mM NADH for ADH1B2; 2.4 mM $NAD^+$ or 1.33 mM NADH for ADH4; 1.2 mM $NAD^+$ or 1 mM NADH for RoDH-4, RDH5 and RDH11.

Esterification assay for human LRAT was performed in the presence of 200 μM α-dipalmitoyl phosphatidyl choline (DPPC) (Sigma-Aldrich) as described previously (Gollapalli and Rando 2003). Twenty-five to fifty micrograms of total microsomal protein was added to the reaction mixture.

The reactions were terminated by the addition of an equal volume of cold methanol. Retinoids were extracted twice with two volumes of hexane, evaporated under a stream of $N_2$, and dissolved in 200 μl of hexane. All retinoid manipulations were performed under dim red light.

Kinetic constants were calculated using the Grafit program (version 5.0, Erithacus Software Limited, Horley, Surrey, UK), and the results were expressed as the mean ± S.E.M of at least three independent determinations.

*HPLC Analysis.* After extraction, retinoids were separated by chromatography on a Spherisorb S3W column (4.6 × 100 mm, Waters) in hexane:methyl-*tert*-butyl ether (96:4, v/v) mobile phase, at a flow rate of 2 ml/min using Waters Alliance 2695 HPLC. Elution was monitored at 350 nm with a Waters 2996 photodiode array, except for esterification assay, where 325 nm was used. Commercially available standards were used to identify the peaks of all-*trans*, 9-*cis*, and 13-*cis* isomers of retinol and retinaldehyde. 9-*cis*-Retinol was synthesized by enzymatic reduction of 9-*cis*-retinaldehyde catalyzed by RDH11 and purified by chromatography as described above. For retinyl esters, all-*trans*-retinyl palmitate was used as a standard.

*Pull-Down Experiments.* Binding of CRBPI to RDH11 was analyzed using either glutathione-Sepharose beads to pull down GST-tagged CRBPI complexed with RDH11-$His_6$ or using anti-$His_6$ antibodies to pull-down $His_6$-tagged RDH11 complexed with untagged apoCRBPI. For GST pull-down experiments, purified RDH11-$His_6$ (3 μg) was incubated with CRBPI-GST fusion protein in phosphate-buffered saline (PBS, 137 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM sodium phosphate, 1.4 mM potassium phosphate) for 1-16 h at room temperature or at 4 ºC. Molar ratio of GST-CRBPI to RDH11-$His_6$ in the mixture varied from 2:1 to 15:1. CRBPI-GST pull-down was performed by incubating the binding mixture with glutathione-Sepharose 4B beads for 30 min on an ice bath. After a quick spin, supernatants were collected and Sepharose beads were washed five times with PBS. Proteins bound to the beads were eluted with 10 mM glutathione in PBS. Proteins in the supernatant and in the eluate were analyzed by SDS-PAGE with subsequent silver staining of the gel.

For immunoprecipitation using monoclonal antibodies against the histidine tag (Clontech, Mountain View, CA, USA), $His_6$-tagged RDH11 was mixed with apoCRBPI and was allowed to bind overnight at 4°C. After the addition of antibodies, the mixture was incubated for 24 h at 4°C and RDH11-$His_6$ – antibody complex was precipitated by binding to protein A-agarose (Pierce Biotechnology, Inc., Rockford, IL, USA) for 1 h at 4°C. Proteins bound to the beads were separated from those remaining in the supernatant by centrifugation and beads were washed five times with PBS. Proteins in the supernatant and on the beads were analyzed by SDS-PAGE as described above.

## RESULTS

*Kinetic comparison of AKR, MDR, and SDR activities with free retinoids.* In view of the recently reported Tween-80 inhibition of ADHs (Martras *et al.,* 2004), we first studied the effect of this detergent on the retinaldehyde reductase activity of AKRs. Like ADHs, AKR1B1 was found to be strongly inhibited by Tween-80. The detergent behaved as an apparent competitive inhibitor with a $K_i$ value of 0.001% (Fig. 10). Therefore, although the presence of 0.02% Tween-80 in the solution provides a reproducible and fast assay for the determination of retinoid activity of AKRs (Crosas *et al.,* 2003), as well as of ADHs (Martras *et al.,* 2004), it is not appropriate for accurate kinetic studies. Thus, the kinetic constants of AKR1B1 were re-evaluated using the detergent-free conditions previously established for microsomal SDRs. Specifically, retinoid substrates were solubilized by sonication with equimolar BSA and the reaction products were analyzed by HPLC after hexane extraction as described under *Experimental Procedures*. In addition, human AKR1C3 of the hydroxysteroid dehydrogenase subfamily of AKRs was included in this study because a retinaldehyde reductase activity was reported for a related enzyme, bovine AKR1C7 (Endo *et al.,* 2001).

In the absence of detergent, the apparent $K_m$ of AKR1B1 for all-*trans*-retinaldehyde was a ~10-fold lower (1.1 μM) (Table 3) than the $K_m$ value previously determined in the presence of Tween-80 (Crosas *et al.,* 2003). Like AKR1B1, AKR1C3 was active toward all-*trans*-retinaldehyde and exhibited a similar $K_m$ value (1.4 μM) (Table 3). The catalytic efficiency ($k_{cat}/K_m$) of AKR1C3 for retinaldehyde reduction was slightly higher than that of AKR1B1 (Table 3). Neither enzyme had significant activity in the oxidative direction with all-*trans*-retinol as a substrate (less than 0.5 $nmol \cdot min^{-1} \cdot mg^{-1}$) (Table 3).

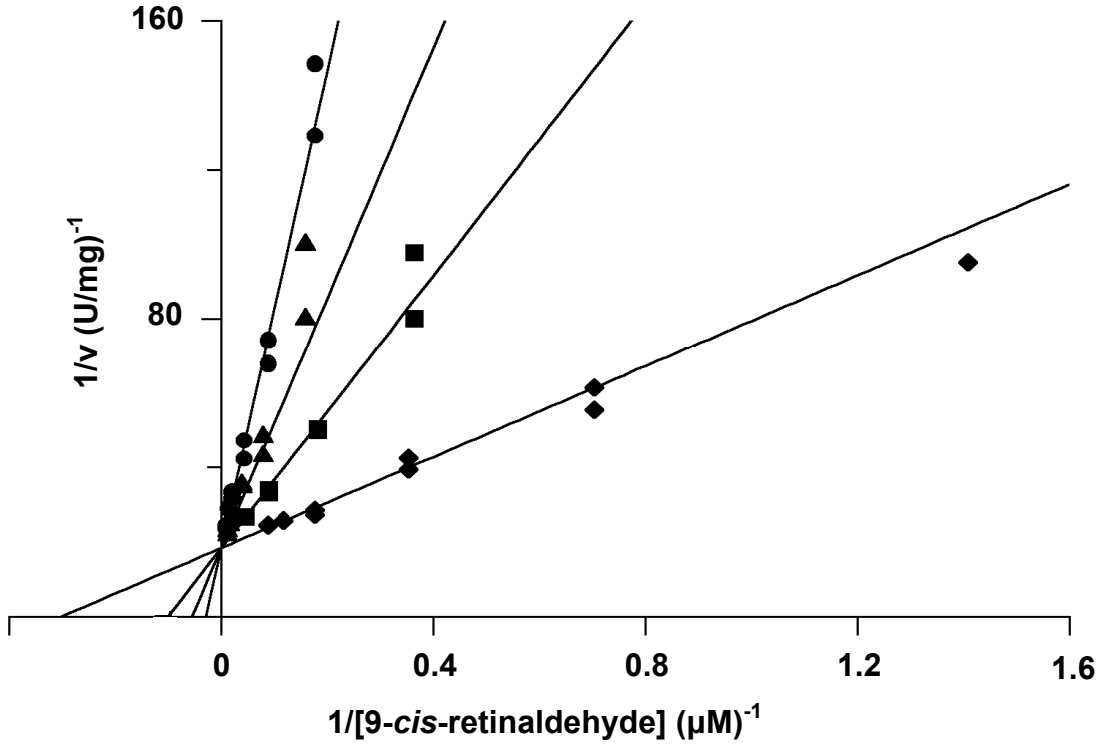


Figure 10**. Lineweaver-Burk plot of inhibition of 9-*cis*-retinaldehyde reductase activity of AKR1B1 by Tween-80.** Retinaldehyde reductase activity was determined in the presence of 0.001% (♦), 0.005% (■), 0.01% (▲) and 0.02% (●) Tween-80, in 0.1 M sodium phosphate, pH 7.5, at 25 ºC and 0.2 mM NADPH.

In contrast, members of the human MDR family, ADH4 and ADH1B2, showed higher catalytic efficiency in the oxidative direction (Table 3). In agreement with previous studies (Yang *et al.,* 1994; Martras *et al.,* 2004), ADH4 was a more catalytically efficient enzyme than ADH1B2. Importantly, both enzymes were found to have a ~100-fold lower $K_m$ values (0.3-0.8 μM) for all-*trans*-retinol and all-*trans*-retinaldehyde than the previously reported values for Tween-solubilized retinoids (Martras *et al.,* 2004).

Kinetic parameters of AKR and MDR oxidoreductases were compared to those of the microsomal SDR enzymes: the $NADP^+$-dependent human RDH11 (Belyaeva *et al.,* 2003) and the $NAD^+$-dependent human RoDH-4 (Lapshina *et al.,* 2003). RDH11 was similar to AKR enzymes, because it preferred $NADP^+$ as a cofactor and was more efficient in the reductive direction (Belyaeva *et al.,* 2003). The catalytic efficiency of microsomal RDH11 for the reduction of all-*trans*-retinaldehyde was at least a 300-fold higher than that of AKR1B1 or AKR1C3 , primarily due to its higher turnover rate (Table 3).

Table 3. **Kinetic constants of purified AKR, MDR and SDR enzymes with Retinoids.**

| Substrate | | | AKR1B1 | AKR1C3 | ADH4 | ADH1B2 | RDH11[a] | RoDH-4 |
|---|---|---|---|---|---|---|---|---|
| all-*trans*-retinol | $K_m$ | (μM) | | | 0.3 ± 0.03 | 0.3 ± 0.1 | 0.6 ± 0.1 | 1.1 ± 0.1 |
| | $k_{cat}$ | ($min^{-1}$) | N.A. | N.A. | 192 ± 6 | 21 ± 1 | 11 ± 0.7 | 1.2 ± 0.02 |
| | *Vmax* | ($nmol \cdot min^{-1} \cdot mg^{-1}$) | N.A. | N.A. | 2400 ± 80 | 260 ± 16 | 300 ± 20 | 35 ± 1 |
| | $k_{cat}/K_m$ | ($mM^{-1} \cdot min^{-1}$) | | | 640000 ± 67000 | 70000 ± 23000 | 18000 ± 3200 | 1100 ± 20 |
| all-*trans*-retinaldehyde | $K_m$ | (μM) | 1.1 ± 0.1 | 1.4 ± 0.3 | 0.8 ± 0.1 | 0.4 ± 0.04 | 0.12± 0.01 | N.D. |
| | $k_{cat}$ | ($min^{-1}$) | 0.35 ± 0.01 | 0.6 ± 0.04 | 302 ± 10 | 5 ± 0.1 | 18 ± 0.5 | --- |
| | *Vmax* | ($nmol \cdot min^{-1} \cdot mg^{-1}$) | 9 ± 0.4 | 15 ± 1 | 3800 ± 120 | 62 ± 1 | 506 ± 13 | --- |
| | $k_{cat}/K_m$ | ($mM^{-1} \cdot min^{-1}$) | 320 ± 30 | 430 ± 100 | 378000 ± 49000 | 13000 ± 1300 | 150000 ± 13000 | --- |

Activities were determined in 90 mM potassium dihydrogen phosphate, 40 mM potassium chloride, pH 7.4, at 37 °C as described under *Experimental Procedures*. [a] Data taken from Belyaeva *et al*., (2003). N.D. Not determined. N.A. No activity was detected or it was less than 0.5 $nmol \cdot min^{-1} mg^{-1}$.

In contrast, RoDH-4 was more similar to ADH enzymes because it preferred $NAD^+$ as a cofactor. The apparent $K_m$ value of RoDH-4 for all-*trans*-retinol, determined using 1 μg/ml of proteoliposomes, was ~1 μM, slightly higher than the $K_m$ values of ADH enzymes (Table 3). However, we noticed that the specific activity of RoDH-4 markedly decreased with increasing concentrations of proteoliposomes. This could indicate that retinol was partially absorbed by proteoliposomes, thus lowering the effective concentration of the solubilized substrate and raising the apparent $K_m$ value for retinol. To determine whether the $K_m$ value of the membrane-bound RoDH-4 depends on the amount of microsomal membranes present in the reaction, we measured the kinetic constants of the enzyme at different concentrations of RoDH-4-containing microsomes. The $K_m$ value for all-*trans*-retinol determined using 0.53 μg/ml of RoDH-4-containing microsomal membranes was 0.3 μM, whereas the $K_m$ value determined using a 10-fold higher concentration of the microsomes (5.3 μg/ml) was 2.6 μM. The inhibitory effect of the membranes showed a competitive pattern with an apparent $K_i$ value of ~1 μg/ml (Fig. 11).

A similar effect was observed in the reductive direction with all-*trans*-retinaldehyde as a substrate. From the inhibition kinetics, the $K_m$ values of RoDH-4 for all-*trans*-retinol and all-*trans*-retinaldehyde were estimated to be 0.29 ± 0.04 μM and 0.34 ± 0.09 μM, respectively. Thus, RoDH-4 appeared to have $K_m$ values for retinoids similar to those of ADHs and reductive SDRs.

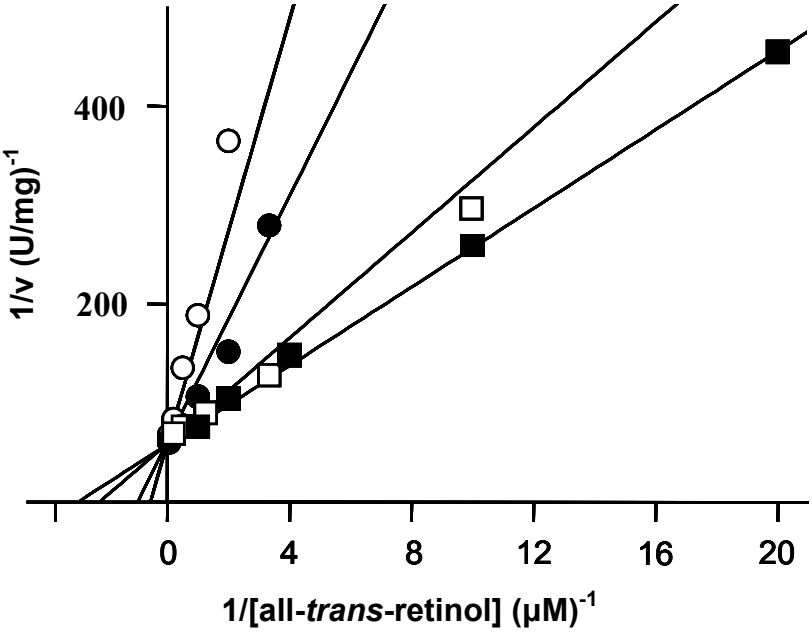


Figure 11. **Lineweaver-Burk plot of the inhibition by microsomal membranes of RoDH-4-catalyzed oxidation of all-*trans*-retinol.** For each reaction, the following amount of microsomal protein was added: 0.13 μg (■), 0.53 μg (□), 2.65 μg (●) and 5.30 μg (○).

*Activity toward CRBPI-bound retinol and retinaldehyde.* Since CRBPI-bound retinol (holoCRBPI) had been proposed to serve as the primary substrate for retinol dehydrogenases, we tested which of the retinoid-active enzymes from AKR, MDR, and SDR superfamilies could oxidize holoCRBPI. As a positive control, we employed human LRAT, which was reported to recognize holoCRBPI as a substrate (Ong *et al.,* 1988; Yost *et al.,* 1988; Herr and Ong, 1992). Recombinant LRAT was prepared by expression in Sf9 cells as described under *Experimental Procedures*. Microsomes containing LRAT were isolated by differential centrifugation and used for activity assays. As shown in Figure 12, LRAT microsomes esterified 2 μM free retinol and 2 μM holoCRBPI with similar efficiency.

In a marked contrast to the efficient esterification of holoCRBPI, the oxidation of holoCRBPI by all three types of human oxidoreductases was much slower than the oxidation of unbound retinol. For example, ADH4 (0.5 μg) produced 2.3 nmol of all-*trans*-retinaldehyde from 2.5 nmol of free retinol in 10 min (Fig. 13A), but only 0.17 nmol of all-*trans*-retinaldehyde from 1 nmol of holoCRBP in 15 min (Fig. 13B). RDH11 (0.1 μg) produced 0.19 nmol retinaldehyde from 2.5 nmol retinol in 15 min, but a 25-fold higher amount of RDH11 (2.5 μg) produced only 0.01 nmol of all-*trans*-retinaldehyde from 5 nmol holoCRBP in 15 min (Fig. 13D). The exact amount of retinaldehyde produced from holoCRBPI varied greatly depending on the enzyme.

These results suggested that either the enzymes utilized CRBPI-bound retinol at a much lower rate than free retinol, or that they did not use bound retinol but the amount of free retinol present in the holoCRBPI preparation was sufficient to support the observed rates. At 2 μM holoCRBPI, the concentration of unbound retinol was calculated as 14 nM, based on the estimated $K_d$ value of 0.1 nM for holoCRBPI (Li *et al.,* 1991; Malpeli *et al.,* 1995). At this concentration, the rate of retinol oxidation by ADH4 was calculated as 107 nmol·min$^{-1}$·mg$^{-1}$ (v = $V_{max}$ × [S] / ($K_m$ + [S]) = 2400 nmol·min$^{-1}$·mg$^{-1}$ × 14 nM/ (300 nM + 14 nM) = 107 nmol·min$^{-1}$ ·mg$^{-1}$). The rate observed in the experiment (22.7 nmol·min$^{-1}$·mg$^{-1}$) did not exceed the predicted rate, suggesting that retinaldehyde production from holoCRBPI could be fully supported by the unbound all-*trans*-retinol in the reaction mixture.

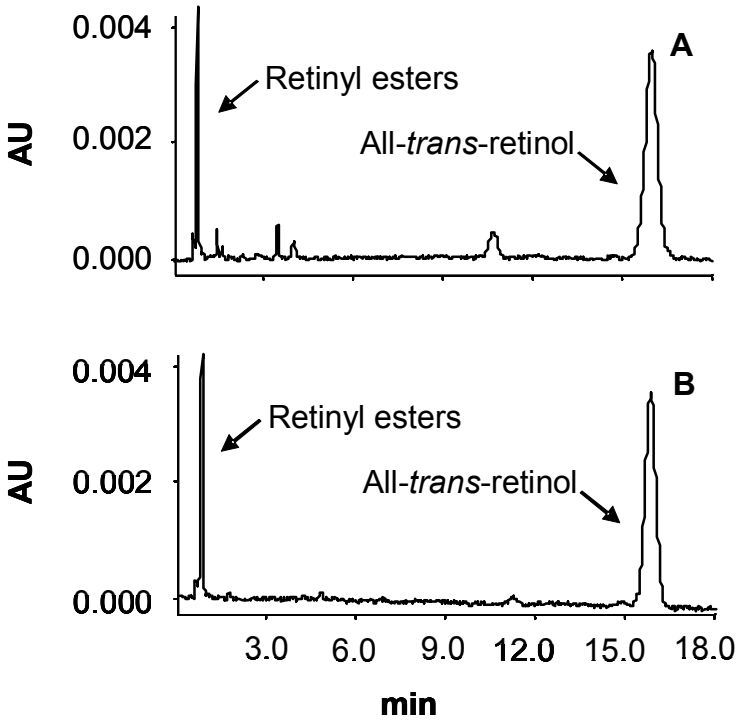


Figure 12. **HPLC elution profiles showing LRAT activity.** Esterification assay for microsomal LRAT was performed with 2 μM all-*trans*-retinol (A) or 2 μM holoCRBPI (B) as a substrate, and 6.2 μg of microsomes containing LRAT. All retinyl esters formed eluted as a single peak.

Consistent with our earlier report (Lapshina *et al.,* 2003), human microsomal RoDH-4 produced retinaldehyde from holoCRBPI (Fig. 13E). However, with the better HPLC separation system employed in the present study, the predominant product of holoCRBPI oxidation was identified as 9-*cis*-retinaldehyde (Fig. 13E, inset, peak 2), not all-*trans*-retinaldehyde. This result confirmed our previous observation that holoCRBPI contains some amount of 9-*cis*-retinol that binds poorly to CRBPI and can be oxidized by *cis*-retinol-active dehydrogenases (Belyaeva *et al.,* 2005). Further support for this finding came from the experiment which demonstrated that 9-*cis*-retinol in holoCRBPI is efficiently oxidized by the microsomal RDH5 (Fig. 13F), an

enzyme more active with 11-*cis*-retinol and 9-*cis*-retinol than with all-*trans*-retinol (Wang *et al.,* 1999). Interestingly, no 9-*cis*-retinaldehyde was detected in the reactions with RoDH-like 3α-HSD, which has no significant activity toward 13-*cis*-retinol (Chetyrkin *et al.,* 2001) or 9-*cis*-retinol (Kedishvili *et al*., unpublished observations). These results suggested that CRBPI did not affect the oxidation of *cis*-retinol but restricted the availability of all-*trans*-retinol for all human enzymes included in this study.

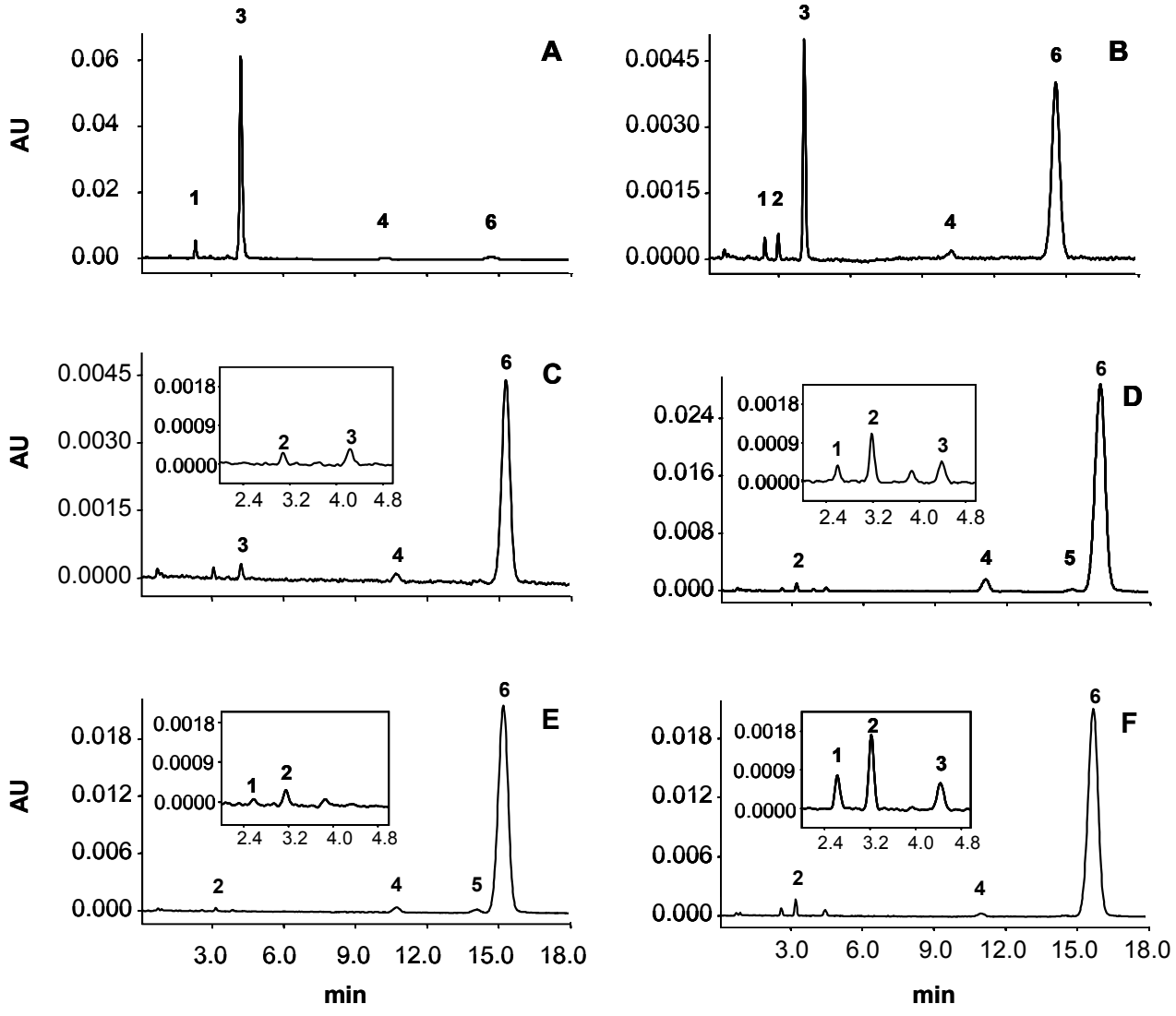


Figure 13. **HPLC elution profiles showing MDR and SDR activities with all-*trans*-retinol and holoCRBPI.** Activities were measured with all-*trans*-retinol, in the free form (A) and bound as holoCRBPI (B-F), using different enzymes: A and B, ADH4; C, ADH1B2; D, RDH11; E, RoDH-4; and F, RDH5. Different isomer peaks could be identified: 1, 13-*cis*-retinaldehyde; 2, 9-*cis*-retinaldehyde; 3, all-*trans*-retinaldehyde; 4, 13-*cis*-retinol; 5, 9-*cis*-retinol; 6, all-*trans*-retinol.

Activities with holoCRBPI were measured as indicated under *Experimental Procedures* for free retinoids but in the absence of BSA. Substrate concentrations were 5 μM for SDRs, RoDH-4 and RDH5, 10 μM for purified RDH11 and 2 μM for ADHs. Protein used was 60 μg for microsomal RoDH-4 and RDH5, 2.5 μg for purified RDH11, and 0.5 μg for ADHs. All reactions were performed in 0.5 ml, and incubated for 15 min, except for ADH4 with free all-*trans*-retinol which was incubated for 10 min. After each reaction, retinoids were extracted and separated as described under *Experimental Procedures*.

The effect of CRBPI on the reduction of all-*trans*-retinaldehyde by ADH4, AKR1B1, and RDH11 was also tested. All-*trans*-retinaldehyde binds to CRBPI with lower affinity ($K_d$ ~50 nM) than all-*trans*-retinol, and the complex of retinaldehyde with CRBPI partially dissociates during gel-filtration (Belyaeva *et al.,* 2005). Therefore, for activity determinations, CRBPI was mixed with retinaldehyde directly in the assay mixture as described previously (Belyaeva *et al.,* 2005). To shift the equilibrium toward bound retinaldehyde, the experiments were carried out in the presence of up to 20-fold molar excess of apoCRBPI over retinaldehyde. As shown in Table 4, the experimental rates of retinaldehyde reduction in the presence of CRBPI obtained with three different enzymes were very close to those predicted based on the calculated concentration of free retinaldehyde in the reactions. For example, the theoretical rate of RDH11 in the reductive direction at 0.5 μM all-*trans*-retinaldehyde, in the presence of 10 μM apoCRBPI, was 20 nmol·min$^{-1}$·mg$^{-1}$. The experimental rate observed under these conditions was exactly the same (Table 4). A similarly good match between the experimental and theoretical rate was observed for AKR1B1, although the rate of ADH4 was lower than the predicted rate (Table 4). Thus, members of the three superfamilies of enzymes appeared to recognize the unbound forms of retinoids both in the oxidative and reductive directions.

Table 4. **Inhibition of all-*trans*-Retinaldehyde Reduction by apoCRBPI.**

| apoCRBPI (μM) | Free retinaldehyde[a] (μM) | Theoretical rate (nmol·min$^{-1}$·mg$^{-1}$)[b] | | | Experimental rate (nmol·min$^{-1}$·mg$^{-1}$) | | |
|---|---|---|---|---|---|---|---|
| | | AKR1B1 | ADH4 | RDH11 | AKR1B1 | ADH4 | RDH11 |
| 0 | 0.5 | 3 | 1400 | 410 | 4 | 900 | 310 |
| 0.5 | 0.14 | 1 | 530 | 270 | 0.7 | 150 | 240 |
| 1 | 0.040 | 0.3 | 180 | 130 | 0.2 | 16 | 130 |
| 2.5 | 0.010 | 0.1 | 55 | 50 | 0.06 | 10 | 60 |
| 10 | 0.006 | 0.05 | 25 | 20 | 0.03 | 5 | 20 |

The experimental rates were determined with 0.5 μM retinaldehyde and different concentrations of apoCRBPI, in 90 mM potassium dihydrogen phosphate, 40 mM potassium chloride, pH 7.4, at 37 °C, using 80 μg, 0.3 μg and 0.19 μg of AKR1B1, ADH4 and RDH11, respectively. For activity measurements in the presence of apoCRBPI, the assay mixture without cofactor was incubated for 15 min at room temperature, to ensure retinaldehyde-CRBPI binding, and the reaction was started by the addition of cofactor. [a]Theoretical amount of free all-*trans*-retinaldehyde was calculated based on the $K_d$ value of 50 nM (Noy, 2000). [b]Theoretical rate was calculated from the Michaelis-Menten equation using the kinetic constants determined by Belyaeva *et al*. (2003) for RDH11 and in the present study for all other enzymes.

To examine a possibility that CRBPI might physically interact with the oxidoreductases, we carried out pull-down assays. Purified RDH11-$His_6$ was incubated with GST-tagged or untagged CRBPI for several hours as described under *Experimental Procedures*. CRBPI was

then pulled down using glutathione-Sepharose beads. Alternatively, RDH11-$His_6$ was pulled down by immunoprecipitation using a monoclonal antibody against the $His_6$ tag. No binding of CRBPI to RDH11-$His_6$ was observed under any conditions tested. Similar results were obtained for ADH and AKR enzymes. Consistent with the lack of protein-protein interaction between the oxidoreductases and CRBPI, 10 μM apoCRBPI had no effect on the ethanol dehydrogenase activity of ADH4, the glyceraldehyde reductase activity of AKR1B1 or the steroid dehydrogenase activity of RoDH-4.

## DISCUSSION

This study represents the first side-by-side analysis of the catalytic properties of human retinoid oxidoreductases from three different enzyme superfamilies: MDRs, SDRs and AKRs. Although each type of retinoid oxidoreductase had been characterized before, the conditions of the assays and the methods used for their analysis were quite different. As shown in this study, Tween-80, frequently employed for solubilization of retinoids, strongly inhibits the retinaldehyde reductase activity of AKR1B1 ($K_i$ ~0.001%). This finding is in agreement with the previous observation that Tween-80 inhibits the retinol dehydrogenase activity of ADH enzymes, resulting in overestimation of their apparent $K_m$ values (Martras *et al.,* 2004). Kinetic analysis in detergent-free conditions carried out in this study now shows that different types of cytosolic and microsomal oxidoreductases all have very similar $K_m$ values for retinol and retinaldehyde. In general, these $K_m$ values are at 1 μM or below. In contrast, different enzymes have dramatically different $k_{cat}$ values. Among the oxidative $NAD^+$-dependent enzymes ADH4 is clearly the most catalytically efficient retinol dehydrogenase, followed by ADH1B2 and RoDH-4. Among the reductive $NADP^+$-dependent enzymes, RDH11 is the most catalytically efficient retinaldehyde reductase, followed by AKR1C3 and AKR1B1.

An important issue that has been addressed by several investigators previously is whether the retinoid-active oxidoreductases utilize retinol or retinaldehyde bound to CRBPI, allegedly the physiological form of the retinoid (Napoli, 1999). Here we have clearly demonstrated that none of the three types of human oxidoreductases appears to oxidize CRBPI-bound retinol or reduce CRBPI-bound retinaldehyde. In contrast, our data indicate that LRAT utilizes holoCRBPI almost as efficiently as free retinol, in agreement with previous observations (Ong *et al.,* 1988; Yost *et al.,* 1988; Herr and Ong, 1992). This outcome is consistent with the results of studies in CRBP

knock-out mice which demonstrated that CRBPI is required mainly for maintenance of normal amount of retinol and its efficient conversion to retinyl esters for storage, but not for retinoic acid synthesis (Molotkov *et al.,* 2004; Matt *et al.,* 2005), and with the observation that several tissues with active retinoic acid synthesis do not express CRBPI (Matt *et al.,* 2005).

Although ADH4 and RDH11 produce a small amount of all-*trans*-retinaldehyde from holoCRBPI, this low level of retinaldehyde production can be supported by the free retinol that dissociates from CRBPI during reaction. This result confirms our previous report that ADH4 does not oxidize CRBPI-bound retinol (Kedishvili *et al.,* 1998). It also shows that ADH4 can oxidize retinol that continuously dissociates from CRBPI. In addition, in spite of the observation that CRBPI exceeds the amount of retinol in liver (Harrison *et al.,* 1987), studies with *Adh1*$^{-/-}$ mutants provide evidence that a small fraction of free retinol normally exists in liver and is continuously oxidized by ADH1 for retinol turnover (Molotkov *et al.,* 2004).

Human RoDH-4 was previously found to oxidize holoCRBPI (Jurukovski *et al.,* 1999; Lapshina *et al.,* 2003). However, re-examination of RoDH-4 activity in the present study revealed that the main product of holoCRBPI oxidation by RoDH-4 is 9-*cis*-retinaldehyde, not all-*trans*-retinaldehyde. As we have established recently, all-*trans*-retinol isomerizes into 9-*cis*-retinol in the presence of CRBPI but not bovine serum albumin when exposed to light (Belyaeva *et al.,* 2005). This isomerization occurs slowly and, therefore, 9-*cis*-retinol is not present in solutions that contain freshly mixed all-*trans*-retinol and CRBPI. These new findings explain why an enzyme closely related to RoDH-4, RoDH-like 3α-hydroxysteroid dehydrogenase (3α-HSD), was previously found to be inactive with holoCRBPI (Lapshina *et al.,* 2003), – the catalytic efficiency of this enzyme was too low to utilize the small amount of available free all-*trans*-retinol in holoCRBPI preparation, whereas 9-*cis*-retinol was a poor substrate for this enzyme.

Our results suggest that both ADH and SDR human enzymes oxidize free but not CRBPI-bound all-*trans*-retinol. CRBPI appears to act as a dispenser of retinol for retinoic acid synthesis. The low $K_m$ values suggest that both enzyme types can effectively bind free retinol. As the free retinol is removed from the medium and oxidized, holoCRBPI dissociates to provide additional free retinol. Although in the presence of holoCRBPI and LRAT the esterification would be a favored reaction, the relative amounts of LRAT and oxidizing enzymes would control the direction and flow of the retinoid metabolism (Fig. 14). In CRBPI knock-out mice the homeostasis of retinoids is altered but the development and the adult functions are normal (Ghyselinck *et al.,* 1999; Matt *et al.,* 2005). Thus, in the absence of CRBPI the control at the

level of retinoid metabolizing enzymes seems sufficient to provide the correct amount of retinoic acid for normal functions.

In rat liver, the major storage site of retinoids, the concentration of CRBPI exceeds that of retinol (7 μM and 5 μM, respectively, Harrison *et al.,* 1987), but in limb buds of chick and mouse embryos retinol levels are much higher than the levels of CRBPI, further suggesting that unbound retinol is required as a source of retinol for retinoic acid synthesis (Scott *et al.,* 1994). In this respect, it is interesting that retinoic acid induces the expression of CRBPI, LRAT, and of the retinoic acid-degrading enzyme CYP26 (reviewed in Ross and Zolfaghari, 2004) (Fig. 14). It appears that there is an automatic feedback loop that signals the cells to slow down retinoic acid production and increase the synthesis of retinyl esters.

An unsolved issue is the relative contribution of cytosolic ADH *versus* microsomal RoDH enzymes to the oxidation of retinol. Both types of enzymes use only free retinol and exhibit similar $K_m$ values, but ADHs are more active in terms of $k_{cat}$, especially ADH4. However, knock-out studies in mice showed that neither ADH enzyme (ADH1, ADH4, or ADH3) is indispensable for retinoic acid synthesis *in vivo*. It is conceivable that ADH4 is required in certain tissues for retinoic acid synthesis during vitamin A deficiency, whereas ADH1 participates in the turnover of retinol in the liver under any dietary vitamin A conditions (Deltour *et al.,* 1999a; Deltour, *et al.,* 1999b; Molotkov *et al.,* 2004).

The role of the less active SDRs in retinoid metabolism *in vivo* is well supported by studies that suggest that mutations in SDR genes are associated with defects in retinoid visual cycle (Yamamoto *et al.,* 1999), with Leber's congenital amaurosis (Janecke *et al.,* 2004), and impaired intestinal development in zebrafish (Nadauld *et al.,* 2005). Surprisingly, SDR enzymes 11-*cis*-RDH and *cis*-retinol/androgen dehydrogenase 1 (CRAD1) appear to be much more efficient than ADH4 at converting 9-*cis*-retinol to 9-*cis*-retinaldehyde in JEG-3 cells (Tryggvason *et al.,* 2001). A relevant role for SDRs may be provided by their microsomal localization. An effective retinoid transfer from CRBPI to membranes exists (Herr *et al.,* 1999) and it is possible that the steady-state levels of unbound retinol are higher in the membrane-rich environment of the microsomal SDR enzymes which may facilitate their utilization of retinoids as compared with cytosolic MDRs.

We have recently recognized that members of the AKR1B subfamily are active retinaldehyde reductases (Crosas *et al.,* 2003), extending the potential contribution to retinoid metabolism to another enzyme type, the AKR superfamily, in addition to MDRs and SDRs. Here we provide further evidence on the participation of AKRs in the reduction of retinaldehyde. We show that AKR1B1 exhibits a lower $K_m$ with this substrate than that previously published

(Crosas *et al.,* 2003). AKR1B1 is the most studied AKR because of its role in several pathologies. Normally, the expression levels of AKR1B1 in most tissues are rather low but this enzyme becomes overexpressed in diabetes, cancer and atherosclerosis (Ramana *et al.,* 2002; Sheetz and King 2002; Zeindl-Eberhart *et al.,* 2004). It is tempting to speculate that its retinaldehyde reductase activity might contribute to disruption of retinoic acid homeostasis and induce pathological changes in tissues where this enzyme is overexpressed.

Moreover, here we have confirmed that members of another AKR1 subfamily, *i.e.* AKR1C, are also active with retinaldehyde. Thus, in addition to bovine AKR1C7 (Endo *et al.,* 2001), AKR1C3 (otherwise known as 3α-HSD type II and 17β-HSD type V) (Penning *et al.,* 2000), which represents a group of AKRs active toward hydroxy- and ketosteroids, also exhibits a retinaldehyde reductase activity. The kinetic parameters of AKR1C3 toward retinaldehyde are similar to those for other substrates analyzed previously (Suzuki-Yamamoto *et al.,* 1999; Penning *et al.,* 2000). Interestingly, AKR1C3 is inducible by retinoic acid (Mills *et al.,* 1998) and its expression is decreased in certain tumor tissues (Lewis *et al.,* 2004).

Both the cytosolic AKRs and microsomal SDR retinaldehyde reductases can easily function in the presence of CRBPI. The data presented in Table 4 demonstrate that while apoCRBPI somewhat inhibits the retinaldehyde reductase activity of the human MDR, SDR, and AKR enzymes, even a 10-fold molar excess of apoCRBPI cannot fully block the reduction of retinaldehyde. This could be due to the limited ability of CRBPI to sequester retinaldehyde compared to retinol. A physiological implication of this observation is that the presence of CRBPI appears to favor the reduction of retinaldehyde to retinol and subsequent esterification of retinol to retinyl esters by LRAT, but it restricts the oxidation of retinol to retinaldehyde by SDR and MDR retinol dehydrogenases.

In conclusion, our work has led us to a new vision of retinoid metabolism. Members of the three enzyme superfamilies, MDRs, SDRs and AKRs, can consistently catalyze the redox reactions of retinoids, although neither of the studied enzymes uses CRBPI-bound retinol/retinaldehyde. CRBPI would have a role in the retinol storage pathway, as opposed to the previously suggested role as a retinol supplier to SDRs in the retinoic acid synthesis pathway. The methodology using BSA as a solubilizing agent, and an HPLC system able to separate isomers of retinol and retinaldehyde, should be employed to acquire comparable kinetics. A remarkable result is that ADHs, SDRs and AKRs display similar submicromolar $K_m$ values with retinoids, making ADH4 the best retinol dehydrogenase ever described because of its very high $k_{cat}$. In addition to the kinetic properties, different factors could regulate the task of each enzyme

in retinoid metabolism inside the cell, such as pattern and level of expression, cofactor specificity or subcellular localization. Thus, more studies *in vivo* should be carried out taking into account members of the three superfamilies. Finally, we cannot exclude the existence of unknown human enzymes able to oxidize CRBPI-all-*trans*-retinol complexes, but the present results and our unpublished data with other members of the three superfamilies suggest that the lack of activity toward CRBPI-bound retinoids is a general feature of these enzymes.

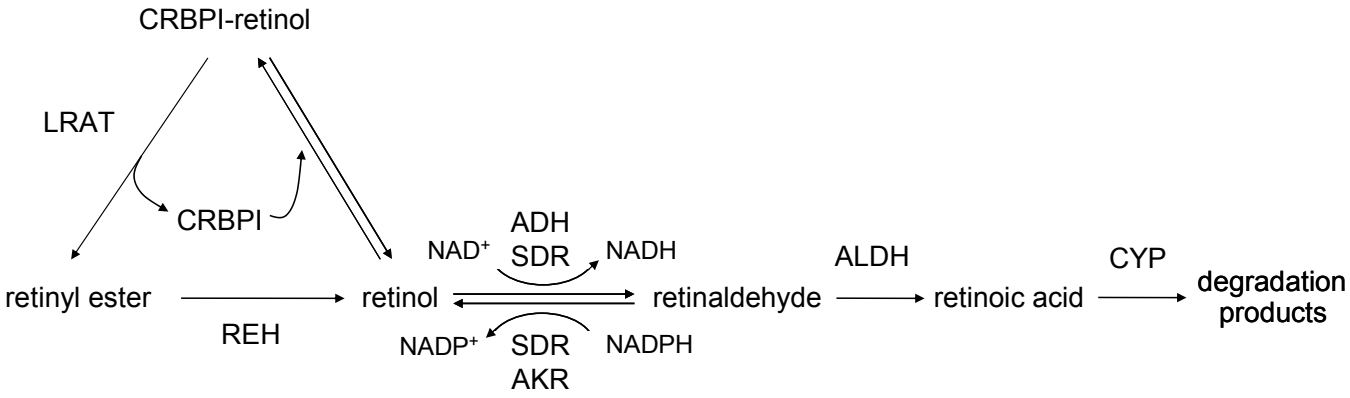


Figure 14. **Schematic diagram of cellular retinoid metabolism.** Levels of CRBPI, LRAT and oxidoreductases influence the retinoid flow either toward the storage pathway or the retinoic acid synthesis. REH, retinyl ester hydrolase; ALDH, aldehyde dehydrogenase; CYP, cytochrome P450.

# CAPÍTOL II

## ESTUDIS ESTRUCTURALS I FUNCIONALS SOBRE L'AKR1B10

## INTRODUCTION

AKR1B10 (human small intestine aldose reductase or aldose reductase-like), a member of the aldo-keto reductase surpefamily (AKR), has been related with different types of human cancer. It has been linked with the development of human hepatocellular carcinoma in different works (Cao *et al.,* 1998; Scuric *et al.,* 1998). Moreover, it was proved that the expression of the enzyme was induced in patients with non-small cell lung carcinoma (NSCLC), mainly linked to tobacco consumption (Fukumoto *et al.,* 2005). Indeed, AKR1B10 has been proposed as a new diagnostic marker for smoking-related NSCLC. Due to the fact that expression of the enzyme was induced in squamous metaplasia (a precancerous lesion), the enzyme could be involved in tobacco-related carcinogenesis.

Retinoids are physiological compounds that participate in several biological processes, such as fetal development or cell proliferation and differentiation. Their biological activity is mainly displayed by retinoic acid, which binds nuclear receptors and controls the expression of different genes (Mangelsdorf *et al.,* 1994). Retinoic acid synthesis is initiated by the oxidation of retinol to retinaldehyde, which is a reversible and the rate-limiting step of process, followed by the oxidation of retinaldehyde to retinoic acid. Three enzyme superfamilies have been related with the first reaction. Short-chain dehydrogenases/reductases (SDR) and medium-chain dehydrogenases/reductases (MDR) share a characteristic Rossmann fold domain, while aldo-keto reductases (AKR) exhibit a $(\alpha/\beta)_8$ barrel structure. Retinoid-active SDRs are membrane-bound enzymes, while MDRs and AKRs are cytosolic. Therefore, this is a clear example of how enzymes with different protein structure and different subcellular localization, converge to the same activity.

$NADP^+$-dependent AKRs are the last group of enzymes identified as retinoid oxidoreductases. Members of the AKR superfamily are enzymes composed of approximately 315 residues, and usually form monomers of 36 kDa (Jez *et al.,* 1997). AKRs reduce a wide variety of aldehydes and ketones, although only three human members of this superfamily have been shown to be active with retinoids. AKR1C3 (3α-HSD type II) shows a low catalytic efficiency with retinaldehyde (430 $mM^{-1}\cdot min^{-1}$, *Capítol I*), similar to that for non-retinoid substrates (Suzuki-Yamamoto *et al.,* 1999; Penning *et al.,* 2000). Interestingly AKR1C3 is inducible by retinoic acid (Mills *et al.,* 1998). Also AKR1B1 (aldose reductase), an enzyme widely studied due to its implication in diabetes (Sheetz and King 2002), shows a low catalytic efficiency with retinaldehyde (320 $mM^{-1}\cdot min^{-1}$, *Capítol I*). In this regard, many inhibitors of

AKR1B1 (ARIs) have been developed with the aim of treating pathologies associated with diabetes, like retinopathy and peripheral neuropathy. Up to now, none of the currently available ARIs has been approved for clinical use (only fidarestat is commercialized in Japan), mainly because of lack of specificity toward the target enzyme. Tolrestat is a widely studied AKR1B1 inhibitor as a potential drug. Clinical trials were done in the past, but it was discarded due to its hepatotoxic effects (Foppiano and Lombardo 1997). Despite of this fact, tolrestat has been a classical AKR1B1 inhibitor used for many studies. Finally, AKR1B10 is a much less studied enzyme (Cao *et al.,* 1998; Hyndman and Flynn 1998), although it has been described as an AKR active with retinoids (Crosas *et al.,* 2003). The ability of AKR1B10 to reduce retinaldehyde has been proposed as a mechanism behind the carcinogenesis process of human NSCLC (Penning 2005). ARI specificity is often determined by comparing its activity against AKR1B1 and AKR1A1, but never before our previous work (Crosas *et al.,* 2003), an ARI had been checked against AKR1B10, and it is not known whether an ARI could affect cellular retinoid metabolism.

Cellular retinol binding protein I (CRBPI) is a cytosolic protein that binds retinol and retinaldehyde with high affinity and has a widespread distribution (Noy 2000). It has been proposed that CRBPI-bound retinol serves as the prime substrate for retinol dehydrogenases responsible for retinoic acid biosynthesis *in vivo* (Napoli 1999). However, in a recent study we proved that none of the human retinoid oxidoreductases tested was able to oxidize bound retinol (*Capítol I*).

Here we have extended the *in vitro* characterization of AKR1B10 with retinoids with a more accurate methodology that does not employ detergent in the assay. Moreover, we studied the role of AKR1B10 in retinoid metabolism in a cell environment, and how it was affected by tolrestat. Finally, we solved the crystal structure of the AKR1B10-$NADP^+$-tolrestat complex, which allowed the prediction of structural determinants for the specificity of AKR1B10 with retinoids, and may facilitate the design of specific inhibitors for AKR1B10.

## EXPERIMENTAL PROCEDURES

*Expression and purification of AKR1B10.* AKR1B10 was purified as described previously for AKR1B1 (*Capítol I*). Briefly, *E. coli* BL21 strain transformed with the plasmid pET16b, that encoded AKR1B10 with a C-terminal His10 tag, was grown in 2xYT medium at 24ºC for 8 h. Protein expression was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Amersham Biosciences, Piscataway, NJ). AKR1B10 was purified using a nickel-

charged chelating Sepharose® Fast Flow resin (Amersham Biosciences, Uppsala, Sweden). The enzyme was eluted by a 0.06-1.0 M imidazole (Sigma-Aldrich, Saint-Louis, MO, USA) gradient in 50 mM Tris-HCl, 100 mM NaCl, pH 8.0. For crystal production, purified protein was incubated overnight with Factor Xa at room temperature. His10 tag and non-digested protein were removed by 30-min incubation with nickel-charged chelating Sepharose® Fast Flow resin at room temperature. Finally, the active enzyme was purified with a Red-Sepharose (Sigma-Aldrich, Saint-Louis, MO, USA) resin and eluted with 2 mM $NADP^+$, 50 mM Tris-HCl, 100 mM NaCl, pH 8.0. Buffer was exchanged to 4 mM $NADP^+$, 25 mM sodium citrate, 20 mM ammonium sulfate, 2 mM DTT, pH 6.0.

*Expression of LRAT in Sf9 cells.* Expression and isolation of microsomes containing LRAT were done essentially as described (*Capítol I*).

*Preparation of apoCRBPI and holoCRBPI.* CRBP type I was expressed in *E. coli* as a C-terminal fusion to a bifunctional tag, consisting of the chitin binding domain (CBD) and intein (CBD-intein). The construct of CRBPI-CBD-intein fusion in the pKYB1 vector (New England Biolabs Inc., Beverly, MA, USA) was expressed in BL21(DE3) cells and purified using the IMPACTTM-CN protein purification system (New England Biolabs Inc.) as described (Belyaeva *et al.,* 2005). CRBPI was released from the intein tag after the induction of the cleavage reaction with 50 mM DTT. To prepare holoCRBPI, an aliquot of purified apoCRBPI was saturated with a 2-fold molar excess of all-*trans*-retinol at room temperature for 1 h. Unbound retinol was removed by gel-filtration on a G50 Sephadex column (Lapshina *et al.,* 2003). The A350/A280 ratio of the holoCRBPI preparation was 1.76. HoloCRBPI was stored in small aliquots at –80º C.

*Enzyme Kinetics.* AKR1B10 standard activity was measured before each kinetic experiment, by using $_{D,L}$-glyceraldehyde as substrate (Crosas *et al.,* 2003). Activity with retinoids in the presence of bovine serum albumin (BSA) was performed in 90 mM potassium phosphate, pH 7.4, 40 mM KCl (reaction buffer), at 37 ºC, in siliconized glass tubes (Gough *et al.,* 1998). Stock solutions of retinoid substrates (Sigma) were prepared in ethanol. For LRAT, dimethylsulfoxide (DMSO, Sigma) was used as a solvent (Gollapalli and Rando 2003). Solvent concentration did not exceeded 1% (v/v) in the reaction mixture and it did not affect the activity rate. Working stock solutions of retinoids were prepared by a 10-min sonication in the reaction buffer in the presence of equimolar delipidated bovine serum albumin. The actual amount of solubilized retinoid was determined based on the corresponding extinction coefficients at the appropriate wavelength. The following molar absorption coefficients in aqueous solution were used: $\varepsilon_{328}$ = 39500 $M^{-1}\cdot cm^{-1}$ for all-*trans*-retinol, $\varepsilon_{400}$ = 29500 $M^{-1}\cdot cm^{-1}$ for all-*trans*-retinaldehyde. Reactions were started by the addition of cofactor and carried out for 5-30 min at

37 ºC. Esterification assay for human LRAT was performed in the presence of 200 μM α-dipalmitoyl phosphatidyl choline (DPPC) (Sigma-Aldrich) as described (Gollapalli and Rando 2003), using from 25 to 50 μg of total microsomal protein. Reactions were terminated by the addition of an equal volume of cold methanol. Retinoids were extracted twice with two volumes of hexane, evaporated under a $N_2$ stream, and dissolved in 200 μl hexane. All retinoid manipulations were performed under dim red light. Kinetic constants were calculated using the Grafit program (version 5.0, Erithacus Software Limited, Horley, Surrey, UK), and the results were expressed as the mean ± S.E.M of at least three independent determinations.

*HPLC Analysis.* After extraction, retinoids were separated by chromatography on a Spherisorb S3W column (4.6 × 100 mm, Waters) in hexane:methyl-*tert*-butyl ether (96:4, v/v) mobile phase, at a flow rate of 2 ml/min using Waters Alliance 2695 HPLC. Elution was monitored at 350 nm with a Waters 2996 photodiode array detector, except for esterification assay, where 325 nm was used. Commercially available standards were employed to identify the peaks of all-*trans*, 9-*cis*, and 13-*cis* isomers of retinol and retinaldehyde. 9-*cis*-Retinol was synthesized by enzymatic reduction of 9-*cis*-retinaldehyde catalyzed by RDH11 and purified by chromatography as described (*Capítol I*). For retinyl esters, all-*trans*-retinyl palmitate was used as a standard.

*Pull-Down Experiments.* Six to thirty μg of AKR1B10-His10 were mixed with up to a five-fold molar excess of CRBPI-GST or apoCRBPI. Samples were incubated for 1 h at room temperature in phosphate-buffered saline in the presence or absence of 1 mM NADPH or $NADP^+$. GST pull-down was performed using glutathione-Sepharose. His-tagged proteins were pulled down by binding to Ni-NTA agarose or by immunoprecipitation using monoclonal antibodies against the His tag (Clontech).

*Cell culture and transfection.* COS-1 cells were grown on 6-well plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Gibco). Incubation was performed at 37°C in a humidified atmosphere containing 5% $CO_2$. For transfection with the plasmid pCMV-HA (Clontech), cells were plated and, after 24 h, treated with LIPOFECTAMINE$^{TM}$ Plus according to the manufacturer's instructions. After 4 h, the fresh medium was added and cells were incubated overnight. Then cells were incubated for 30 min with retinoid and/or tolrestat. Control cells were incubated with the same proportion of ethanol than that used for retinoid and tolrestat incubations, never exceeding 0.1% of the medium volume. After incubation, cells were rinsed twice in ice-cold phosphate-buffered saline and harvested by scraping into 200 μl 0.002% (v/v) SDS. Cell suspensions were stored frozen at -80

ºC. The thawed suspensions were sonicated on ice to complete cell lysis. Retinoids were extracted and analyzed as described above.

Cellular retinoid conversion was expressed as percentage of retinaldehyde that had been reduced to retinol (100 × pmol cell-retinol/(pmol cell-retinol + pmol cell-retinaldehyde)) or retinol that had been oxidized to retinaldehyde. We did not take account of retinoids present in the culture medium.

*Western blotting.* Polyclonal antiserum specific against the AKR1B10 C-terminal sequence QSSHLEDYPFDAEY was kindly provided by Dr. T. Geofrey Flynn. Anti-haemagglutinin (anti-HA) (12CA5) antibody was from Roche. Goat anti-rabbit IgG (H+L) peroxidase-conjugated antibody was from Bio-Rad (Hercules, CA, U.S.A.).

*Crystallization and structure determination.* Crystals were grown at 20ºC by vapor diffusion using the hanging drop method. One µl of protein solution (18 mg/ml) was mixed with 1 µl of polyethylene glycol (PEG) 6000, 100 mM sodium cacodylate, pH 9.0 (precipitant solution). The hanging drop was equilibrated with 0.8 ml of precipitant solution. Crystals belonged to the hexagonal space group P61 (a = b = 89.1 Å, c = 78.4 Å, α = β = 90.0º, γ = 120.0º), with one molecule in the asymmetric unit. Data was collected at 100 Kº on a ADSC Q4R CCD detector in the European Synchroton Radiation Facility (ESRF). Diffraction data were integrated and scaled using DENZO and SCALEPACK (Otwinowski and Minor 1997). The structure was solved by molecular replacement using the coordinates of PDB entry 1FRB (Wilson *et al.,* 1995), as a model with MOLREP. Subsequent refinement was carried out using REFMAC (Collaborative Computational Project 1994) and manual model building in O (Jones *et al.,* 1991). Figures were performed using PYMOL (http://www.pymol.org).

## RESULTS

*Redefining AKR1B10 kinetic constants for retinoids.* Since cofactor specificity for AKR1B10 had been never reported, we first determined the $K_m$ values for NADH and NADPH using glyceraldehyde as a substrate. As expected, the enzyme showed a clear preference for NADPH with $K_m$ = 10.6 ± 1.9 µM, while NADH was unable to saturate the enzyme.

Recently, we showed that the presence of detergent Tween-80 in the activity assays caused an overestimation of the $K_m$ values of MDRs and AKRs for retinoids (Martras *et al.,* 2004), and that the actual values were similar to those of SDRs (*Capítol I*). In the present work, *in vitro* activities of AKR1B10 with retinoids were reevaluated using a new methodology in the absence

of detergent (Gough *et al.,* 1998). AKR1B10 exhibited for both all-*trans*-retinaldehyde and all-*trans*-retinol, $K_m$ values below 1 μM (Table 5), 30-fold lower than those obtained by using Tween-80 in the assay (Crosas *et al.,* 2003). These values are in the range of those reported for human retinoid oxidoreductases of the SDR type with the same methodology (Belyaeva *et al.,* 2005, *Capítol I*). Moreover the enzyme showed the highest $k_{cat}$ value with retinaldehyde (27 $min^{-1}$) within AKRs, comparable to the best value ever reported for any $NADP^+$-dependent retinaldehyde reductase (RDH12 showed a $k_{cat}$ = 36 $min^{-1}$, Belyaeva *et al.,* 2005). In terms of catalytic efficiency ($k_{cat}/K_m$) with retinaldehyde, AKR1B10 showed values two orders of magnitude higher than other human AKRs, mainly due to its high $k_{cat}$.

Table 5. **Kinetic constants of human AKRs with retinoids.**

| Substrate | | | AKR1B10 | AKR1B1[a] | AKR1C3[a] |
|---|---|---|---|---|---|
| all-*trans*-retinol | $K_m$ | (μM) | 0.4 ± 0.1 | | |
| | $k_{cat}$ | ($min^{-1}$) | 7.2 ± 0.3 | N.A. | N.A. |
| | *Vmax* | (nmol·$min^{-1}$·$mg^{-1}$) | 190 ± 8 | N.A. | N.A. |
| | $k_{cat}/K_m$ | ($mM^{-1}$·$min^{-1}$) | 18000 ± 4500 | | |
| all-*trans*-retinaldehyde | $K_m$ | (μM) | 0.6 ± 0.1 | 1.1 ± 0.1 | 1.4 ± 0.3 |
| | $k_{cat}$ | ($min^{-1}$) | 27 ± 1 | 0.35 ± 0.01 | 0.6 ± 0.04 |
| | *Vmax* | (nmol·$min^{-1}$·$mg^{-1}$) | 700 ± 40 | 9 ± 0.4 | 15 ± 1 |
| | $k_{cat}/K_m$ | ($mM^{-1}$·$min^{-1}$) | 45000 ± 7600 | 320 ± 30 | 430 ± 100 |

Activities were determined in 90 mM potassium dihydrogen phosphate, 40 mM potassium chloride, pH 7.4, at 37°C, as described under *Experimental procedures*. [a] Data taken from *Capítol I*. N.A., no activity was detected or it was lower than 0.5 nmol/min·mg.

*Activity of AKR1B10 towards CRBPI-bound retinoids.* We studied whether the enzyme was able to oxidize all-*trans*-retinol or reduce all-*trans*-retinaldehyde bound to CRBPI. As a positive control, we employed human LRAT (Fig. 12, in *Capítol I*), which was reported to recognize holoCRBPI as a substrate (Ong *et al.,* 1988; Yost *et al.,* 1988; Herr and Ong 1992). We demonstrated that AKR1B10 was unable to use CRBPI-bound retinoids as substrates. Even though activity was observed in presence of CRBPI (Fig. 15B), this activity could be fully supported by the amount of free retinoid that continuously would dissociate from the CRBPI-retinoid complex. Thus, under the conditions that were used in the experiment (2 μM holoCRBPI), the concentration of free retinol was calculated to be 14 nM. At this concentration, the rate of retinol oxidation by AKR1B10 was calculated as 6.4 nmol·$min^{-1}$·$mg^{-1}$ ($v = V_{max} \times [S] /$

($K_m$ + [S]) = 190 nmol·min$^{-1}$·mg$^{-1}$ × 14 nM/ (400 nM + 14 nM) = 6.4 nmol·min$^{-1}$·mg$^{-1}$). The observed rate in the experiment (0.3 nmol·min$^{-1}$·mg$^{-1}$) was much lower than the predicted rate. Therefore, CRBPI almost inhibited completely the oxidation of retinol. In the case of the retinaldehyde reductase activity of AKR1B10, despite of using up to 20-fold excess of CRBPI over retinaldehyde, the enzyme always showed significant retinaldehyde reductase activity (Table 6). This difference between retinol dehydrogenase and retinaldehyde reductase activities in the presence of CRBPI probably was due to the large difference in the dissociation constant ($K_d$) values of CRBPI for retinol and retinaldehyde (0.1 nM and 50 nM, respectively, Li *et al.,* 1991; Malpeli *et al.,* 1995).

To exclude a possibility that CRBPI might physically interact with AKR1B10, we carried out pull-down assays. No binding of apoCRBPI or holoCRBP to AKRB10-His6 was observed either in the presence or in the absence of cofactors, $NADP^+$ or NADPH. Consistent with the lack of protein-protein interaction between the enzyme and CRBPI, 10 μM apoCRBPI had no effect on the glyceraldehyde reductase activity of AKR1B10 (O.V. Belyaeva, personal communication).

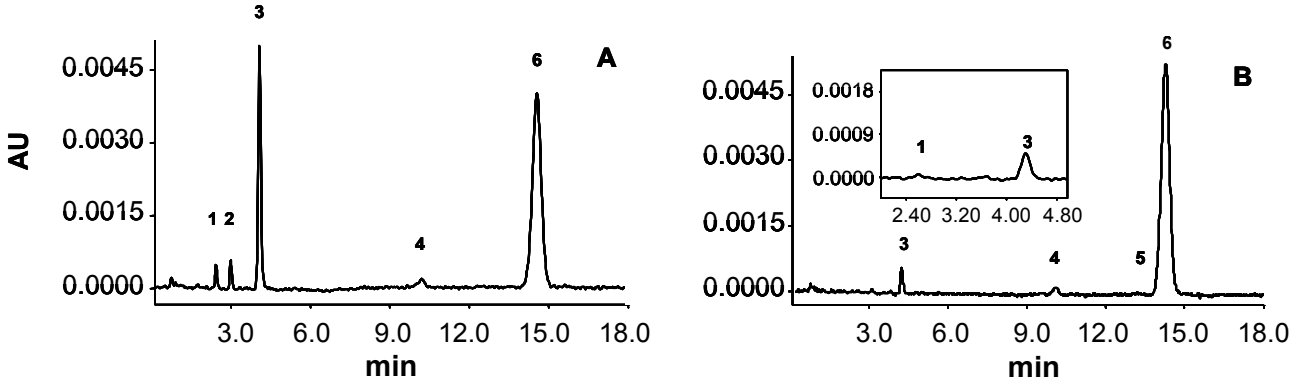


Figure 15. **HPLC elution profiles showing AKR1B10 activity with all-*trans*-retinol and holoCRBPI.** Activity was measured with all-*trans*-retinol, in the free form (A) and bound as holoCRBPI (B). Different isomer peaks could be identified: 1, 13-*cis*-retinaldehyde; 2, 9-*cis*-retinaldehyde; 3, all-*trans*-retinaldehyde; 4, 13-*cis*-retinol; 5, 9-*cis*-retinol; 6, all-*trans*-retinol.

Activiy with holoCRBPI was measured as indicated in *Experimental procedures* for free retinoids but in the absence of BSA. Reactions were performed with 2 μM substrate, in 0.5 ml, and incubated for 15 min. After reaction, retinoids were extracted and separated as described in *Experimental procedures*.

Hence, our data suggested that AKR1B10 did not employ any retinoid bound to CRBPI, although it showed a stronger activity with retinaldehyde than with retinol in the presence of the binding protein.

Table 6. **Inhibition of all-*trans*-retinaldehyde reductase activity of AKR1B10 by apoCRBPI.**

| apoCRBP (μM) | Calculated free retinaldehyde[a] (μM) | Rate (nmol·min$^{-1}$·mg$^{-1}$) | |
|---|---|---|---|
| | | Theoretical[b] | Experimental |
| 0 | 0.5 | 330 | 300 |
| 0.5 | 0.14 | 140 | 90 |
| 1 | 0.040 | 50 | 30 |
| 2.5 | 0.010 | 15 | 11 |
| 10 | 0.006 | 7 | 5 |

The experimental rates were determined with 0.5 μM retinaldehyde and different concentrations of apoCRBPI, in 90 mM potassium dihydrogen phosphate, 40 mM potassium chloride, pH 7.4, at 37° C, using 0.5 μg AKR1B10. In the activity measurements in the presence of apoCRBPI, the assay mixture without cofactor was incubated for 15 min at room temperature, to ensure retinaldehyde-CRBPI binding, and the reaction was started by addition of cofactor. [a]Theoretical amount of free all-*trans*-retinaldehyde was calculated based on the $K_d$ value of 50 nM (Malpeli *et al.,* 1995). [b]Theoretical rate was calculated from the Michaelis-Menten equation using the kinetic constants determined in the present study.

*AKR1B10 as a retinaldehyde reductase in the cell environment.* After showing a relevant retinaldehyde reductase activity of AKR1B10 *in vitro* (Crosas *et al.,* 2003, and present work) we investigated the involvement of AKR1B10 in retinoid metabolism *in vivo*. COS-1 cells were transfected with the plasmid pCMV-HA containing AKR1B10 cDNA (COS/AKR1B10-HA). The optimal amount of DNA employed for cell transfection (2 μg) was determined by Western blot analysis using specific antibodies against the hemagglutinin tag or AKR1B10 (Fig. 16A). Interestingly, the specific antibody detected a small amount of endogenous monkey homolog of AKR1B10 in wild-type cells (non transfected, COSwt).

Wild-type cells (COSwt), cells transfected with empty vector (COS/pCMV-HA) and COS/AKR1B10-HA were incubated for 30 min with 10 μM all-*trans*-retinaldehyde, and their intracellular retinoid content was analyzed. Under these conditions, production of retinyl esters or retinoic acid was undetectable. Control cells displayed a marked ability to reduce retinaldehyde to retinol since they converted approximately 25% of intracellular retinaldehyde. However cells transiently expressing AKR1B10 showed a two-fold higher reduction rate, reaching approximately 55% of conversion (Fig. 16B).

Interestingly, incubation with retinol did not produce a significant increase in intracellular retinaldehyde content, even in cells transfected with vector containing AKR1B10 cDNA. Thus, *in vivo*, AKR1B10 only was able to metabolize retinaldehyde, acting as a retinaldehyde reductase, and causing a decrease of the intracellular supply of retinaldehyde for retinoic acid synthesis.

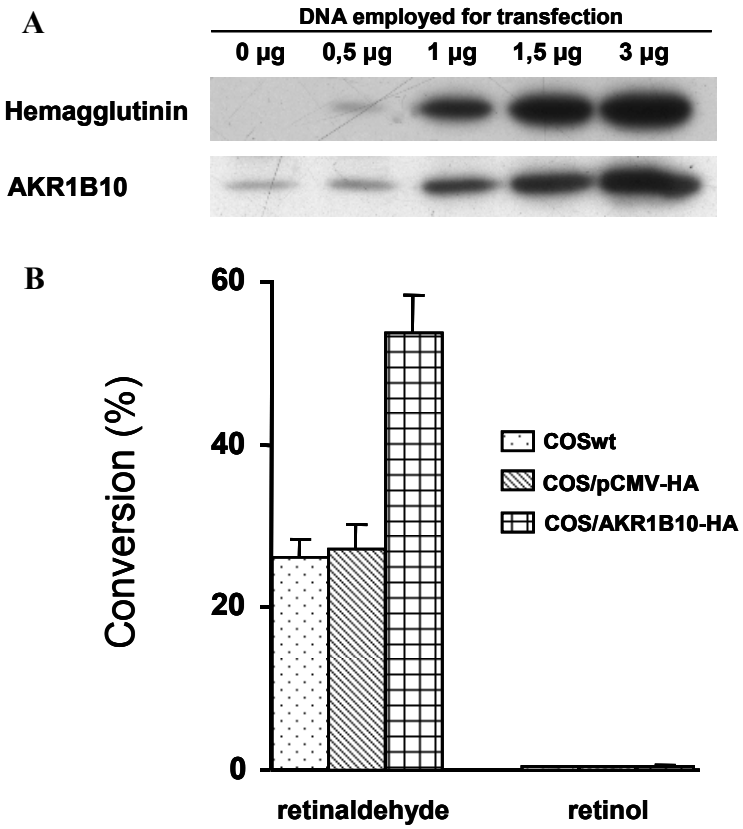


Figure 16. **Retinoid metabolism in COS-1 cells transiently expressing AKR1B10-HA. A** Western blot analysis of COS-1 cell cultures transfected with different amounts of pCMV/AKR1B10-HA vector. Western blot was developed employing specific antibodies against hemagglutinin tag or AKR1B10. **B** Cellular retinoid content was measured by HPLC as described in *Experimental procedures*, after incubating cells for 30 min with 10 µM retinaldehyde or retinol. No retinyl esters or retinoic acid production was observed. Thus, only retinol or retinaldehyde were measured, and data was expressed as percentage of conversion of the retinoid uptaken by cells (reduced retinaldehyde or oxidized retinol).

*Tolrestat inhibition of retinaldehyde reductase activity in vitro and in vivo.* In a previous work we tested the effect of tolrestat on retinaldehyde reductase activity of AKR1B1 and AKR1B10 using Tween-80 in the assay (Crosas *et al.,* 2003). Similar Ki values were obtained for the two enzymes, but they were 10-fold higher than those obtained using AKR1B1 and glyceraldehyde (Vander Jagt *et al.,* 1990). Here, we reevaluated the inhibitory effect of the drug on the retinaldehyde reductase activity of AKR1B10 measured in the absence of detergent. With the new methodology, the IC50 obtained was approximately 10 nM (Fig. 17A), a value similar to that previously published for AKR1B1 with glyceraldehyde (Ehrig *et al.,* 1994).

Then we tested the effect of tolrestat on the retinaldehyde reductase activity of cells expressing AKR1B10 (Fig. 17B). When cells were incubated with 1 µM tolrestat, the retinaldehyde reductase activity contributed by the enzyme *in vivo* was reduced by approximately 50%. Activity due to AKR1B10 was completely blocked by incubating cells with 10 µM tolrestat. Thus, tolrestat was revealed as a potent inhibitor of AKR1B10 *in vitro* and *in vivo.*

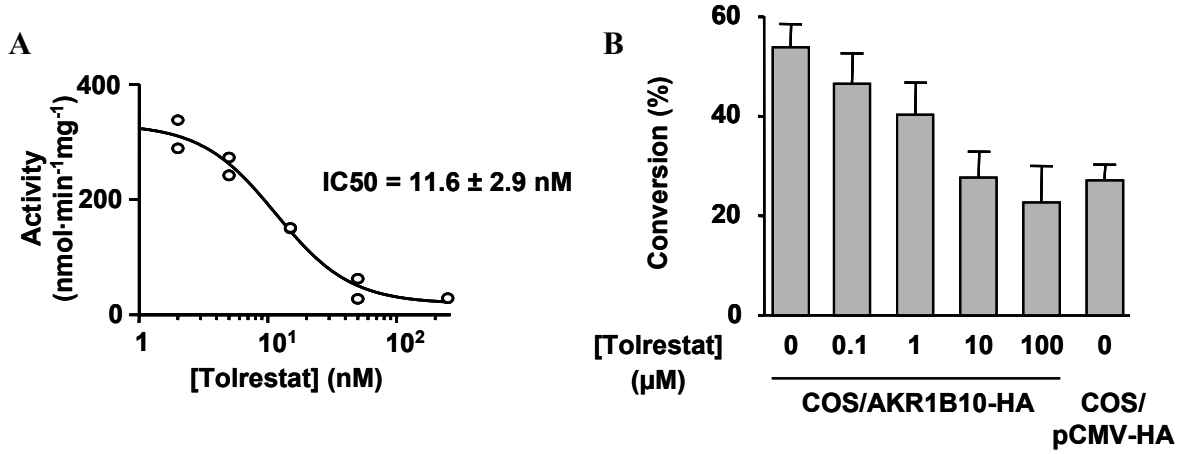


Figure 17. ***In vitro* and *in vivo* effect of tolrestat on retinaldehyde reductase activity. A** Determination of tolrestat IC50 for retinaldehyde reductase activity of AKR1B10. Activity was mesured using 0.5 μM retinaldehyde in 90 mM potassium dihydrogen phosphate, 40 mM potassium chloride, pH 7.4, at 37° C. **B** Tolrestat inhibition of cellular AKR1B10 activity. COS-1 cells transfected with pCMV/AKR1B10-HA were incubated with 10 μM all-*trans*-retinaldehyde and different amounts of tolrestat. Retinoid content was mesured as described in *Experimental procedures*, and conversion was expressed as percentage of the retinaldehyde uptaken by the cells that was reduced. Conversion for COS-1 cells transfected with empty vector is shown as a control. Results are expressed as the mean ± S.E.M. of at least three determinations.

Table 7. **Crystal data collection and refinement statistics.**

| | |
|---|---|
| **Data collection** | |
| Space group | *P*61 |
| Unit cell paramenters (Å, º) | a = 89, b = 89, c = 78<br>α = 90, β = 90, γ = 120 |
| Resolution (Å) | 55.05-1.25 (1.30-1.25) |
| Unique reflections | 92,151 |
| Completeness (%) | 99.5 |
| Mosaicity (º) | |
| $R_{merge}$ (%) | 5.6 (40) |
| Multiplicity | |
| <I/σ(I)> | 5 (2) |
| **Refinement** | |
| Resolution range (Å) | 20-1.25 |
| Reflections (%) | 92,151 (345) |
| R factor(%) | 13.00 |
| R free (%) | 15.68 |
| Average B (Å$^2$) | 14.26 |
| Numer of atoms | 6034 |
| Protein atoms | 5,352 |
| $NADP^+$ molecules | 1 |
| Tolrestat molecules | 1 |
| Waters molecules | 610 |
| RMSD from ideal values | |
| Rmsd bond (Å) | 0.022 |
| Rmsd angle (º) | 2.094 |
| Ramachandran plot | |
| Most favoured (%) | 90.4 |
| Additional allowed (%) | 9.2 |
| Generously allowed (%) | 0.0 |
| Disallowed (%) | 0.4 |

*Crystal structure of AKR1B10 complexed with $NADP^+$ and tolrestat.* We solved the crystal structure of AKR1B10 complexed with cofactor and tolrestat at 1.25-Å resolution (Fig. 18, Table 7). As expected, the structure of AKR1B10 showed the $(\alpha/\beta)_8$ barrel typical of the AKR superfamily. $NADP^+$ was bound in the side of the barrel (Fig. 18A), perpendicularly to the central cavity, where the carboxyl group of the inhibitor was located. Thus, the catalytic site was placed in the center of $(\alpha/\beta)_8$ barrel.

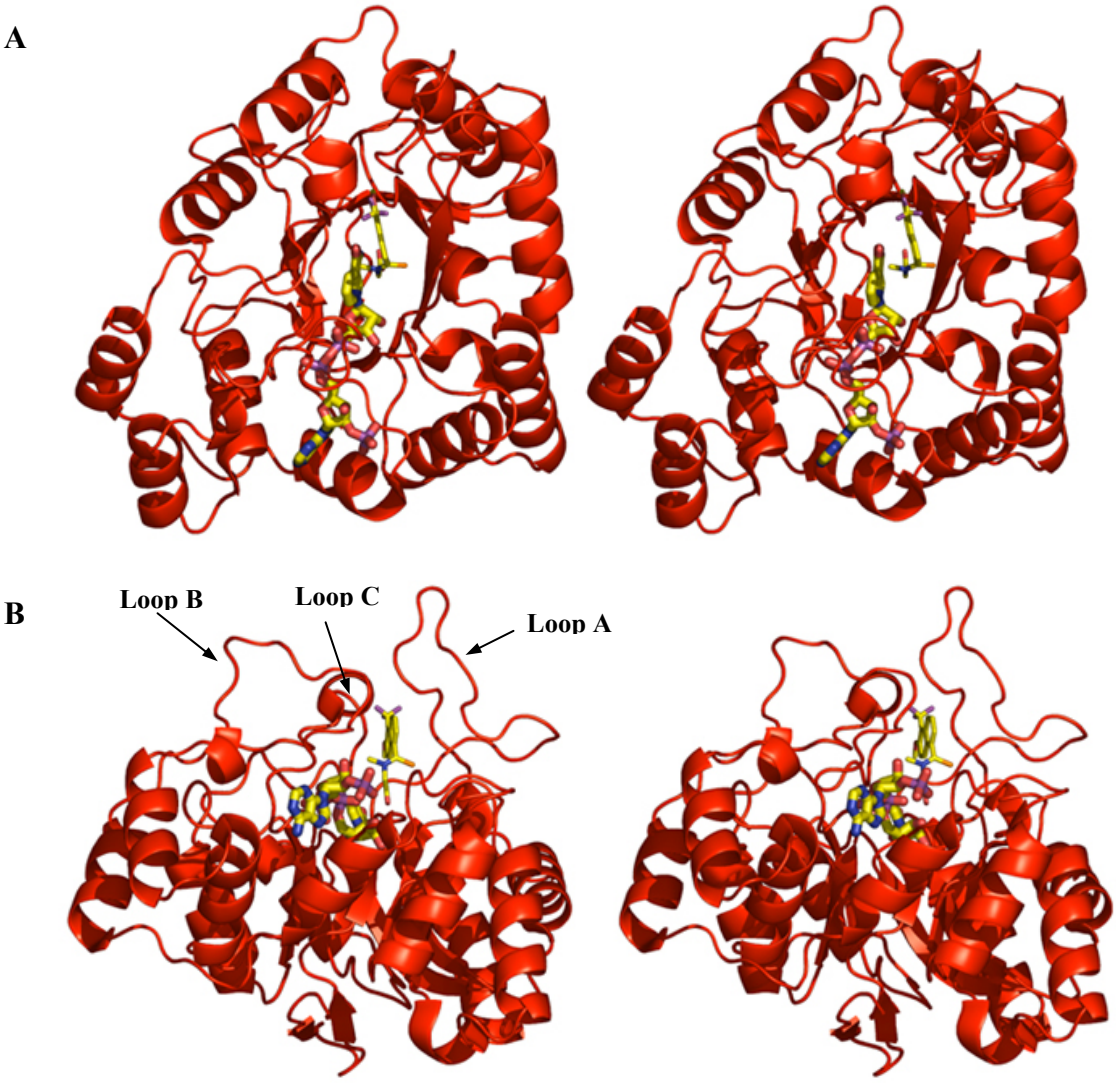


Figure 18. **Stereo view of crystal structure of AKR1B10 complexed with $NADP^+$ and tolrestat. A** View from the top of the $(\alpha/\beta)_8$ barrel. The catalytic site is located in the center of the barrel. **B** View of the $(\alpha/\beta)_8$ barrel after rotating 90º. Cofactor approaches the catalytic site from one side of the barrel, while tolrestat enters the barrel from the upper face.

The high-resolution data allowed us to determine tolrestat position accurately (Fig. 19). The carboxyl group of tolrestat was positioned at catalytical distances from the C4 atom of cofactor (3.27 Å), and the hydroxyl group of Tyr49 (2.63 Å).

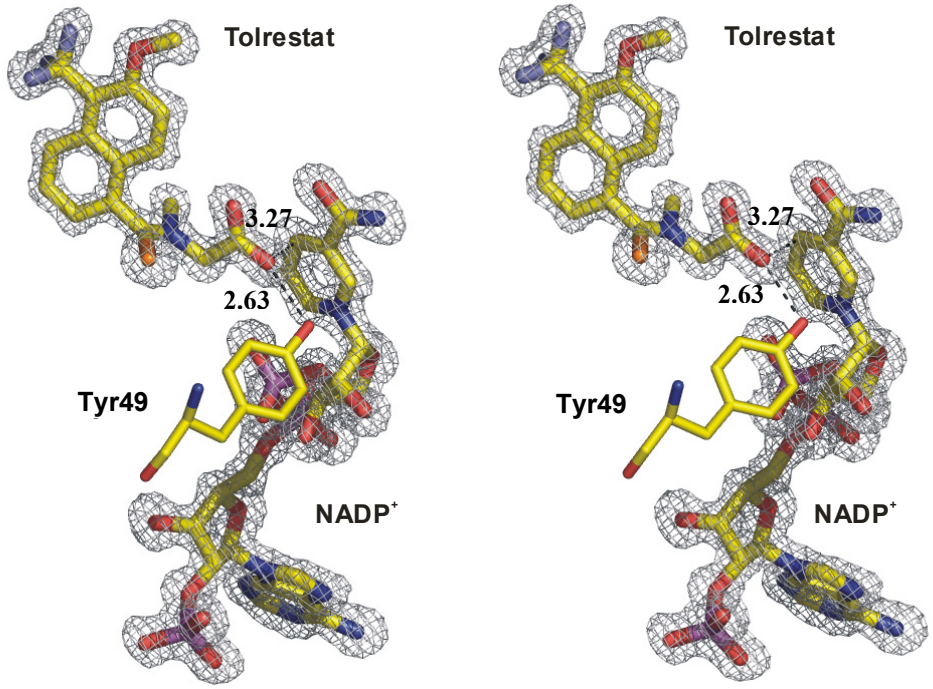


Figure 19. **Stereo view of OMIT map of NADP$^+$ and tolrestat.** Electron density proves the high quality of our data. Main atomic distances in the catalytic site are showed. Thus, the carboxyl group of tolrestat is located at 2.63 Å from OH of Tyr 49 and 3.27 Å from C4 atom of NADP$^+$.

A list of interactions between tolrestat and AKR1B10 was obtained by a LIGPLOT representation for the crystal structures of AKR1B10 (Fig. 20A) and AKR1B6 (porcine orthologous of human AKR1B1, the crystal structure of AKR1B1 complexed with tolrestat is not available) (Fig. 20C). Tyr49, His111 and Trp112 established hydrogen bonds with O2 and O3, while Trp21, Val48, Trp80, Phe116, Phe123, Trp220, Cys299, Val301, Gln303 and NADP$^+$ composed a hydrophobic pocket. Only positions 301 and 303 differed between the two enzymes. Val301 is replaced by Leu in AKR1B6, keeping van der Waals interaccions with tolrestat. Thus, the only relevant change between the two structures is the substitution of Gln303 by Ser in AKR1B6. In the AKR1B6-NAPD$^+$-tolrestat structure, Ser303 is establishing a hydrogen bond with F1 of tolrestat. This is lost in the AKR1B10 complex, but Gln303 remains interacting with F1 of tolrestat, which is consistent with a similar effect of the inhibitor on AKR1B1 and AKR1B10 kinetics.

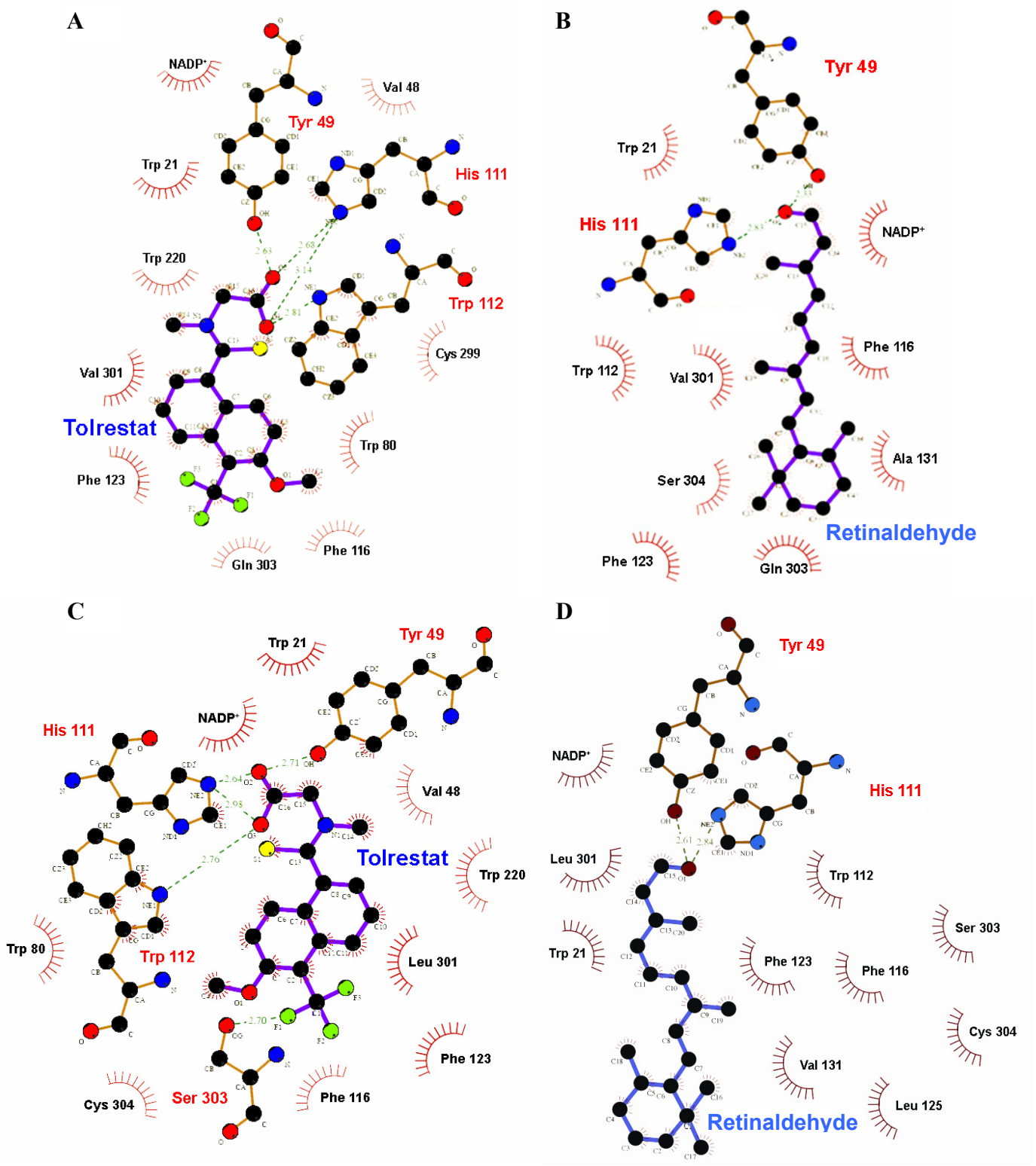


Figure 20. **LIGPLOT (Wallace *et al.*, 1995) describing interactions in the substrate-binding site. A and C** Interactions of tolrestat molecule in the AKR1B10-NADP$^+$-tolrestat and AKR1B6-NADP$^+$-tolrestat complexes, respectively. **B and D** Interactions of all-*trans*-retinaldehyde predicted in the models, of AKR1B10-NADP$^+$-all-*trans*-retinaldehyde and AKR1B1-NADP$^+$-all-*trans*-retinaldehyde complexes, respectively.

*Modeling of retinaldehyde binding.* Our crystal structure allowed us to predict a model of a ternary complex between AKR1B10-$NADP^+$ and all-*trans*-retinaldehyde, and compare it with a model of the AKR1B1-$NADP^+$-all-*trans*-retinaldehyde complex (Figs. 21B and 22B, respectively). All-*trans*-retinaldehyde fitted nicely into both substrate-binding pockets, in a similar way than the inhibitor did. Predicted retinaldehyde-binding pocket was a large and extremely hydrophobic cavity. Since retinaldehyde is a larger molecule than tolrestat, the cyclohexene ring of retinoid fully occupied the internal face of loops A and C, thus increasing the number of contacts in comparison with the inhibitor. Sequence identity between AKR1B1 and AKR1B10 is aproximately 70%, but their kinetic constants are quite different for retinoids (Table 5). Our model allows to hypothesize some structural reasons to explain such differences.

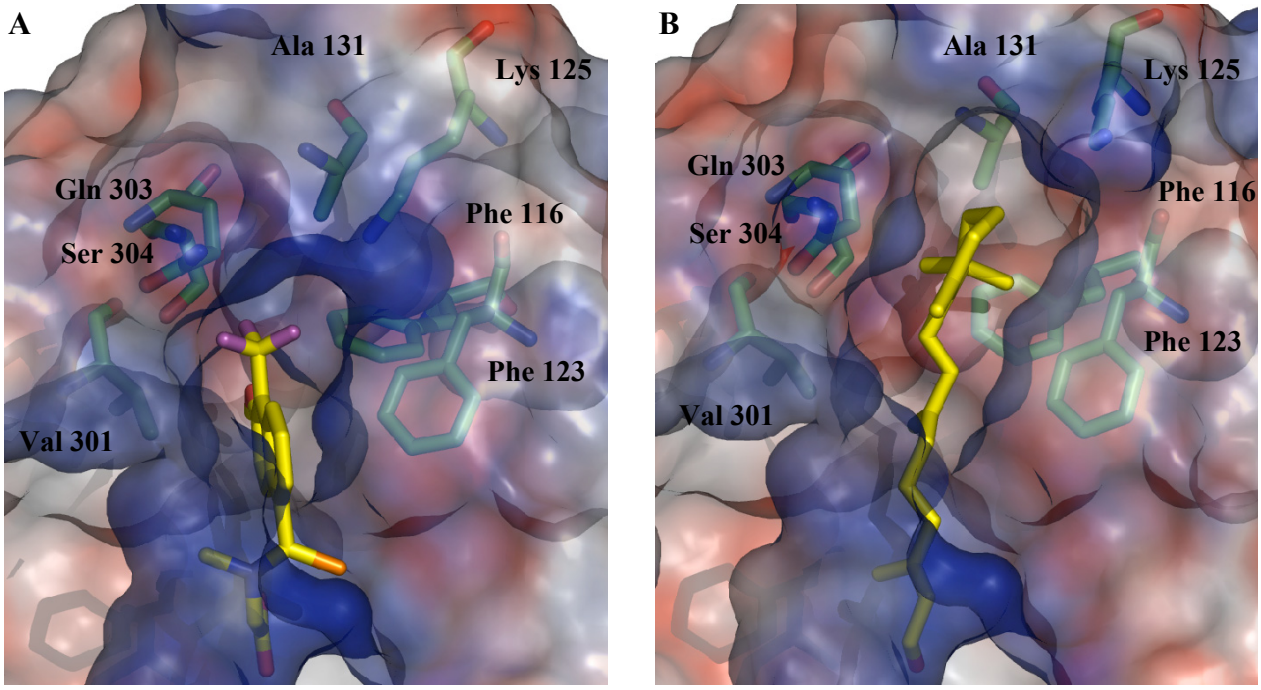


Figure 21. **Model of all-*trans*-retinaldehyde docked into the AKR1B10 binding pocket. A** Representation of tolrestat-binding pocket in the AKR1B10-$NADP^+$-tolrestat crystal. **B** Representation of retinaldehyde-binding pocket predicted by our model. The molecular surface is coloured according to the local electrostatic potential as calculated with the program PYMOL (http://www.pymol.org). All-*trans*-retinaldehyde binds in a similar manner than tolrestat. Residues around the substrate define a highly hydrophobic and well adjusted pocket, protecting the retinaldehyde molecule from the polar solvent. In both structures all residues occupy similar positins except Lys125 side chain which, in the model (B), has to switch 5.4 Å to enlarge the binding pocket and allow retinaldehyde binding.

LIGPLOT representations for all-*trans*-retinaldehyde binding to AKR1B10 and AKR1B1 (Figs. 20B and 20D, respectively) provided a list of interactions which are characteristic for each enzyme. Most aminoacid residues involved in retinaldehyde binding are conserved in the two structures. Thus, the inner part of the cleft (defined by Trp21, Tyr49, His111, Trp112, Phe116,

Phe123) is practically identical between AKR1B1 and AKR1B10. Main differences between the two models were found in the external part where all the residues are substituted: Lys125Leu (loop A), Ala131Val (loop A), Val301Leu (loop C), Gln303Ser (loop C) and Ser304Cys (loop C), for AKR1B10 and AKR1B1, respectively. Modifications in residues 301, 303 and 304 did not seem to alter van der Waals interactions between each enzyme and retinaldehyde. By comparing the two models (Figs. 21B and 22B), it is easy to identify residues 125 and 131 as those that are subjected to a larger shift. The most relevant is position 125, which in AKR1B10 is a Lys residue exposed to the solvent and it does not interact with the ligand, while in AKR1B1 is a Leu residue that appears to pack retinaldehyde, blocking its release.

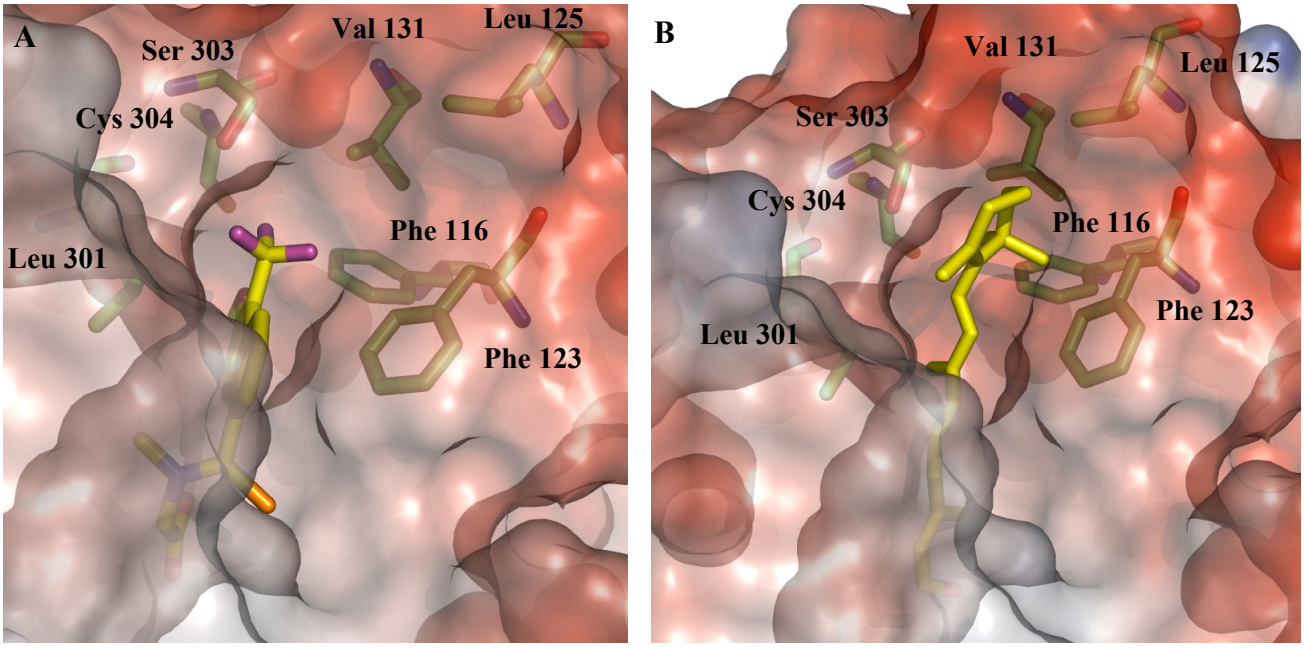


Figure 22. **Model of all-*trans*-retinaldehyde docked into the AKR1B1 binding pocket. A** Representation of tolrestat-binding pocket in the AKR1B6-$NADP^+$-tolrestat crystal. **B** Representation of retinaldehyde-binding pocket predicted by our model. The molecular surface is coloured according to the local electrostatic potential as calculated with the program PYMOL (http://www.pymol.org). All-*trans*-retinaldehyde binds in a similar manner than tolrestat. Residues around the substrate define a highly hydrophobic and well adjusted pocket, protecting the retinaldehyde molecule from the polar solvent. Leu125 keeps a similar position in the two structures.

## DISCUSSION

Different works had proved the relationship between AKR1B10 overexpression and different types of human cancer (Cao *et al.,* 1998; Scuric *et al.,* 1998; Zeindl-Eberhart *et al.,* 2004). Recently, AKR1B10 was revealed as a new diagnostic marker for human NSCLC, where

the enzyme is highly overexpressed, mainly in smoking-related cases (Fukumoto *et al.,* 2005). Little is known about the physiological function of AKR1B10, but since it is highly active towards retinoids (Crosas *et al.,* 2003), it may participate in the retinoic acid synthesis pathway. These findings suggest that AKR1B10 may be involved in the carcinogenesis process that leads to NSCLC, and other types of human cancer, through the modification of retinoic acid homeostasis (Penning, 2005). Thus, in the present work we further studied the role that AKR1B10 could play in retinoid metabolism. After the recent discovery that Tween-80 inhibits competitively AKR1B1 (*Capítol I*), a first objective was to reevaluate the kinetic constants of AKR1B10 for retinol-retinaldehyde conversion in the absence of detergent. The new methodology employed in the present work had already been used for a previous comparative study between human AKRs, ADHs and SDRs, and provided more reliable data (*Capítol I*). Here, AKR1B10 was shown as a novel enzyme within the group of proteins that could participate in the first step of retinoic acid synthesis *in vivo*. The $K_m$ value for all-*trans*-retinaldehyde determined for AKR1B10 (~0.6 μM) was in the same order of magnitude as those values described previously for other human enzymes involved in retinoid metabolism (*Capítol I*). On the other hand, the $k_{cat}$ value (27 $min^{-1}$) was close to the highest value found for any NADPH-dependent retinaldehyde reductase (Belyaeva *et al.,* 2005). Thus, AKR1B10 showed a catalytic efficiency of 45000 $mM^{-1}\cdot min^{-1}$ for retinaldehyde reduction, a value 100-fold higher than those for other retinoid-active AKRs. Although the enzyme exhibited activity with both retinol and retinaldehyde, it showed clear preference for aldehyde reduction. Furthermore, AKR1B10 displayed a high specificity for NADPH over NADH, supporting its function as a reductase. Moreover, the enzyme is unique within the human AKRs due its ability to oxidize retinol *in vitro*.

CRBPI-bound retinol had been proposed by some authors (Napoli, 1999) as the main molecular form that supplies retinol for retinoic acid synthesis *in vivo*. Our study proved clearly that AKR1B10 was unable to metabolize retinol or retinaldehyde bound to CRBPI. Hence, AKR1B10 can be added to the growing list of enzymes that do not use holoCRBPI as a substrate, a group that includes all the human retinol oxidoreductases tested up to now (Belyaeva *et al.,* 2005, and *Capítol I*). Taken altogether, *in vitro* data point to a role of AKR1B10 in cellular retinol metabolism. Moreover, the fact that none of the retinol dehydrogenases tested, from any of the three superfamilies, is active with holoCRBPI supports that free retinol is the actual substrate in the oxidative pathway of retinoic acid synthesis. Retinol binding to CRBPI may have a role of protecting the retinol from unnecessary oxidation or faciliting esterification by LRAT.

The present study constitutes the first report where the retinol oxidoreductase activity for a human AKR has been examined in a cell environment. AKR1B10 efficiently reduced retinaldehyde, and thus it diminished the cellular retinaldehyde pool, which is necessary for retinoic acid production. In contrast, the enzyme was unable to oxidize retinol to retinaldehyde *in vivo*, consistent with its cofactor specificity. Thus, it can be concluded that AKR1B10 acts as an efficient retinaldehyde reductase *in vivo*.

*In vitro* and *in vivo* activity of AKR1B10 was inhibited by tolrestat, a well known AKR1B1 inhibitor designed for diabetes treatment. Tolrestat inhibited retinaldehyde reductase activity of AKR1B10 with the same IC50 as that reported for the inhibition of glyceraldehyde reduction by AKR1B1 (IC50 ~ 10 nM, Ehrig *et al.,* 1994). Also, in COS-1 cells, tolrestat inhibited AKR1B10, similarly to the effect on AKR1B4 (the rat orthologous of AKR1B1) in rat vascular smooth muscle cell culture (IC50 ~ 1 μM, Ramana *et al.,* 2002). Therefore, tolrestat proved to be an efficient AKR1B10 inhibitor. It could have potential applications in the cancer treatment field, where the decrease in retinaldehyde reductase activity of AKR1B10 could raise retinoic acid levels. On the other hand, AKR1B1 inhibitors, designed to prevent sorbitol accumulation, had also been tested against AKR1A1 with the aim to exclude effects on other metabolic pathways. The inhibition of AKR1B10 here demonstrated could explain why some ARIs show nonspecific effects, since they inhibit AKR1A1 much weakly than AKR1B10 (tolrestat inhibits glyceraldehyde reduction by AKR1A1 with a Ki ~ 2 μM, Vander Jagt *et al.,* 1990). Hence, our results suport that ARIs should also be routinely tested against AKR1B10 to prevent potential side effects due to alteration of retinoic acid synthesis.

The AKR1B1 structure is well established (Wilson *et al.,* 1992), and complexes with many inhibitors had been reported (Wilson *et al.,* 1993; Urzhumtsev *et al.,* 1997; El-Kabbani *et al.,* 2004). Also AKR1A1 had been crystallized in a complex with tolrestat (el-Kabbani *et al.,* 1997). With the novel structure of AKR1B10 complexed with tolrestat, here reported, we analyzed the structural features common to the enzymes of the superfamily, and we tried to identify the structural differences that could be used to design specific AKR1B10 inhibitors, of possible interest for cancer treatment. The structural differences may also explain the unique specificity of AKR1B10 with retinoids. AKR1B10 presented the typical $(\alpha/\beta)_8$ barrel, with the catalytic site in the middle. Tolrestat is bound in a deep cleft, the substrate-binding pocket, essentially establishing the same interactions as in AKR1B6 (the porcine orthologous of AKR1B1). This explains the nearly identical inhibitory effect of tolrestat with AKR1B1 and AKR1B10.

Different AKRs showed a wide spectrum of activity toward retinoids. While AKR1A2 was not active (Crosas *et al.*, 2003), AKR1B1 exhibited low $k_{cat}$ values, and AKR1B10 proved to be

the best retinaldehyde reductase, with a catalytic efficiency 100-fold higher than that of AKR1B1. Hence, because of the high sequence identity between AKR1B1 and AKR1B10, few residue differences should explain their distinct specificity with retinoids. In the model of AKR1B10-$NADP^+$ complexed with all-*trans*-retinaldehyde, the substrate was docked in a productive manner inside the binding pocket, with the carbonyl group located at the proper distance for catalysis between the C4 atom of cofactor and the OH of Tyr49. Retinaldehyde is a larger molecule than tolrestat, resulting in its cyclohexene ring fully interacting with the most external part of the cleft, mainly loops A and C. By comparing AKR1B1-$NADP^+$-all-*trans*-retinaldehyde and AKR1B10-$NADP^+$-all-*trans*-retinaldehyde models, we identified position 125 (Lys in AKR1B0 and Leu in AKR1B1) as the prime substitution that may be involved in the distinct kinetic behaviour of the two enzymes with retinaldehyde. In the AKR1B10-$NADP^+$-all-*trans*-retinaldehyde model, Lys125 adopted a conformation with its side chain pointing away from the substrate and being more exposed to the solvent, while in the AKR1B1 model, this residue was interacting with all-*trans*-retinaldehyde. In the AKR1B1 model, the exit from the binding pocket appeared to be blocked by Leu125 side chain, which should move away to allow product release. It is assumed that, for the best substrates, the rate-limiting step in AKRs is cofactor dissociation (Grimshaw *et al*., 1990; Kubiseski *et al*., 1992; Grimshaw *et al*., 1995). The low $k_{cat}$ value of AKR1B1 with retinaldehyde supports the notion that another step is limiting, probably the dissociation of the retinol product. It is conceivable that residue 125, Lys in AKR1B10, is responsible for the high $k_{cat}$ of this enzyme with retinaldehyde.

Further work needs to be undertaken to explain the high catalytic efficiency of AKR1B10 towards all-*trans*-retinaldehyde. This could also reveal novel strategies to design new pharmacological compounds suitable to inhibit AKR1B1 or AKR1B10 specifically. Retinoid derivatives could represent a new family of more specific inhibitors with applications in diabetes and cancer.

# DISCUSSIÓ GENERAL

Estudis anteriors del nostre grup (Crosas *et al.,* 2003; Martras *et al.,* 2004) havien demostrat que el Tween-80, un detergent emprat en els assajos cinètics d'ADH i AKR amb retinoides, provocava un efecte d'inhibició competitiva en les ADH estudiades. En aquesta Tesi s'ha observat que el Tween-80 també és un inhibidor de les AKR. En condicions d'absència de detergent, hem determinat que els valors de $K_m$ per ADH i AKR, són entre un i dos ordres de magnitud inferiors als mesurats anteriorment amb Tween-80. Així, ADH, AKR i SDR presenten valors de $K_m$ per als retinoides molt similars (0,1-1 μM), i pròxims a les concentracions fisiològiques d'aquests compostos. A més, també s'ha demostrat que cap de les oxidoreductases humanes estudiades, representatives de les tres superfamílies, no reconeix com a substrats els retinoides units a CRBPI. Anteriorment, s'havia observat que algunes SDR sí utilitzaven holoCRBPI. Ara, però, sabem que aquells resultats eren artefactes deguts a la presència de petites quantitats de 9-*cis*-retinol lliure en les preparacions d'holoCRBPI. Amb el nou mètode d'anàlisi de retinoides, capaç de diferenciar els isòmers de retinol i retinal, s'ha pogut determinar amb exactitud que cap dels enzims no pot oxidar o reduir retinoides units a CRBPI. Un valor de $K_m$ inferior, i la capacitat d'oxidar retinol unit a CRBPI, eren els dos principals arguments per als qui defensaven un paper principal de les SDR en la primera etapa de síntesi de l'àcid retinoic *in vivo*, la interconversió de retinol i retinal. Ara, podem afirmar que el principal tret cinètic diferenciador entre els enzims de les tres superfamílies enzimàtiques es troba en els valors de $k_{cat}$. Els nostres resultats prediuen que les ADHs, i sobretot l'ADH4, són enzims importants en el metabolisme de retinoides per la seva elevada $k_{cat}$. Altres aspectes, com la localització subcel·lular o el patró d'expressió, contribuiran també a determinar el paper que cada enzim pugui jugar *in vivo*.

S'han dut a terme nombrosos estudis per caracteritzar el paper fisiològic de SDR i ADH en el metabolisme de retinoides. Diferents treballs amb ratolins *knock-out* (Deltour *et al.,* 1999a; Deltour *et al.,* 1999b; Driessen *et al.,* 2000; Shang *et al.,* 2002; Molotkov *et al.,* 2002a; Molotkov *et al.,* 2004) han provat la seva participació *in vivo*. Fins al moment, però, no hi ha hagut cap treball similar amb AKR. En aquesta Tesi, s'ha abordat per primera vegada l'estudi de la funció de l'AKR1B10 en el metabolisme de retinoides en un entorn cel·lular. En cèl·lules COS-1, l'AKR1B10 és capaç de reduir retinal però, a diferència de les seves propietats *in vitro*, no oxida retinol. Per tant, aquests estudis recolzarien un paper de les AKR en la reducció del retinal, d'acord també amb la seva especificitat per NADPH, en la primera etapa de la via metabòlica de l'àcid retinoic.

Un estudi recent (Fukumoto et al., 2005) va revelar que AKR1B10 podia tenir importància en el desenvolupament de NSCLC humà (que representa el 80% dels casos de càncer de pulmó). L'enzim es va trobar sobreexpressat en un gran nombre de pacients, principalment en fumadors. Fins i tot, l'expressió de l'enzim es va trobar alterada en estadis precancerosos. Aquestes observacions van fer postular que AKR1B10 podia estar afectant la síntesi d'àcid retinoic, i així participar en la carcinogènesi de NSCLC humà (Penning 2005). A més, també s'ha observat que l'enzim es sobreexpresa en altres tipus de càncers humans, com el HCC (Cao *et al.,* 1998; Scuric *et al.,* 1998; Zeindl-Eberhart *et al.,* 2004). Ara, demostrada l'elevada capacitat de l'enzim per reduir retinal tant *in vitro* com *in vivo*, s'afegeixen nous arguments per proposar una possible alteració en el flux de la via de síntesi de l'àcid retinoic, en casos on els nivells d'AKR1B10 són elevats. L'enzim podria revertir l'oxidació de retinol a retinal cel·lular i, per tant, limitar el flux global de la via. L'elevada expressió d'AKR1B10 en teixits cancerosos resultaria en una disminució dels nivells d'àcid retinoic cel·lular, afectant processos com la diferenciació i proliferació cel·lular.

L'estudi d'inhibidors d'AKR1B1, amb aplicacions farmacològiques en el tractament de la diabetis, té una llarga trajectòria. Són molts els inhibidors desenvolupats, però encara no se n'han obtingut amb suficient eficiència i lliures d'efectes secundaris. Amb aquest treball, afegim dos nous aspectes a tenir en compte en l'estudi dels ARI. En primer lloc, demostrem que el tolrestat, un inhibidor que en estudis *in vitro* s'havia mostrat específic d'AKR1B1, també inhibeix AKR1B10, tant *in vitro* com *in vivo*. Això podria explicar en part els efectes secundaris que presenta l'ús del fàrmac en humans. A partir de l'exemple del tolrestat, caldria desenvolupar nous ARI capaços de distingir entre els dos enzims. Finalment, l'experiència acumulada en el disseny d'ARI podrà ser útil per a la recerca d'inhibidors específics per a AKR1B10. Aquests podrien tenir aplicacions en la prevenció o el tractament de diferents tipus de càncers humans.

L'estructura tridimensional obtinguda per al complex AKR1B10-$NADP^+$-tolrestat, es revela com una potent eina de treball. En primer lloc, seria la base pel disseny de nous inhibidors capaços de distingir entre d'AKR1B1 i AKR1B10. Però també, per a la identificació dels determinants estructurals de l'activitat retinal reductasa d'algunes AKR, principalment de la mateixa AKR1B10. Les interaccions amb el tolrestat són pràcticament idèntiques en AKR1B1 i AKR1B10. En la part més externa del centre d'unió al substrat és on es troben un major nombre de diferències entre les dues estructures. A partir dels models obtinguts per als complexos amb tot-*trans*-retinal, s'observa com l'anell de ciclohexè interacciona amb aquesta regió, compresa entre els llaços A i C. Els contactes característics que l'anell estableix en cada model podrien explicar les propietats cinètiques pròpies de cada enzim. En concret, proposem el residu 125 com

a posició crítica per a la unió del retinal. Així, inhibidors basats en l'estructura de retinoides podrien donar lloc a una nova família de compostos capaços de distingir entre AKR1B1 i AKR1B10, doncs és clar que els retinoides són reconeguts de forma diferent per cadascun dels enzims, tal i com mostra la important diferència entre els respectius valors de $k_{cat}$.

# CONCLUSIONS

1. El detergent Tween-80, utilitzat per dissoldre retinoides en els assajos enzimàtics, causa una inhibició competitiva aparent de l'activitat ADH i AKR, de la qual en resulten uns valors artefactualment elevats de $K_m$ per als retinoides.

2. Els estudis cinètics en absència del detergent proven que els enzims de les tres superfamílies d'oxidoreductases actives amb retinoides (AKR, ADH i SDR) tenen valors de $K_m$ similars, entre 0,1 i 1 μM. Les diferències cinètiques es concentren principalment en els valors de $k_{cat}$.

3. Els enzims dependents de NADH (ADH i algunes SDR) mostren preferència per l'oxidació del tot-*trans*-retinol, sent l'ADH4 la que presenta una major eficiència catalítica, seguida de l'ADH1B2 i la RoDH4.

4. Els enzims dependents de NADPH (AKR i algunes SDR) mostren una clara preferència per la reducció del tot-*trans*-retinal sobre l'oxidació del retinol. Els enzims més actius són la RDH11, seguida de l'AKR1B10.

5. Cap dels enzims assajats de les tres superfamílies d'oxidoreductases no es mostra actiu amb retinoides units a CRBPI, el que qüestiona resultats obtinguts en treballs anteriors i el paper de la CRBPI en la via de síntesi de l'àcid retinoic.

6. En cél·lules COS-1, l'AKR1B10 actua com a retinal reductasa, però no com a retinol deshidrogenasa. Fa disminuir, així, el contingut de tot-*trans*-retinal intracel·lular.

7. El tolrestat és un potent inhibidor de l'activitat retinal reductasa d'AKR1B10 tant *in vitro* com *in vivo*, i el seu efecte inhibidor es similar al que produeix sobre AKR1B1.

8. S'han obtingut cristalls del complex AKR1B10-$NADP^+$-tolrestat que difracten raigs X, el que ha permès recollir dades de fins a una resolució de 1,25 Å.

9. AKR1B10 presenta una estructura tridimensional similar a la resta d'AKR, basada en el plegament de barril $(\alpha/\beta)_8$. La majoria de diferències amb AKR1B1 es troben localitzades en la part més externa del centre d'unió al substrat, principalment en els llaços A i C.

10. La unió del $NADP^+$ i el tolrestat es dóna de forma molt similar, i amb pràcticament les mateixes interaccions, en AKR1B10 i AKR1B1, el que explica perquè l'inhibidor no és capaç de discriminar entre els dos enzims.

11. L'estructura d'AKR1B10 ha permès obtenir el model del complex AKR1B10-$NADP^+$-tot-*trans*-retinal. La molècula de retinal encaixa perfectament en el centre actiu de l'enzim, situant-se a una distància òptima de la tètrada catalítica i del cofactor per a una catàlisi productiva.

12. En comparar els models d'unió del retinal per a AKR1B10 i AKR1B1, es poden identificar interaccions úniques per a cadascun dels complexos, que podrien explicar les propietats cinètiques pròpies de cada enzim. Els residus 125 i 131 es proposen com a crítics per a la unió correcte del retinal.

13. L'estructura del complex amb tolrestat i els models amb retinoides podrien servir per millorar el disseny de nous inhibidors més específics per a AKR1B1 i AKR1B10, amb possibles aplicacions en el tractament de complicacions secundàries de la diabetis i diferents càncers humans.

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