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Characterization of Rat Testes Mitochondrial Retinoylating System and Its Partial Purification

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Retinoylation (retinoic acid acylation), a posttranslational modification of proteins occurring in a variety of eukariotic cell lines both in vivo and in vitro, was studied in rat testes mitochondria. all-*trans*-Retinoic acid, a highly active form of vitamin A in inducing cellular differentiation, is incorporated covalently into proteins of rat testes mitochondria. The maximum retinoylation activity of rat testes mitochondrial proteins was 21.6 pmoles mg protein^{-1} 90 min^{-1} at 37°C. The activation energy was 44 kJ mol^{-1} from 5 to 37°C. The retinoylation activity had a pH optimum of 7.5. The retinoylation process was specific for the presence of ATP, ADP, and GTP (even if only 30% of the control). The half saturation constant (K_m) was 0.69 μM for all-*trans*-retinoic acid, while the inhibition constant (K_i) was 1.5 μM for 13-*cis*-retinoic acid. Retinoylation was not inhibited by high concentrations of myristic acid (MA) and palmitic acid (PA), indicating that retinoylation and acylation reactions involved different rat testes mitochondrial proteins. The ATP or CoASH saturation curves of retinoylation reaction showed sigmoidal behavior with apparent half saturation constants ($K_{0.5}$) of 6.5 mM ATP and 40.6 μM CoASH. On SDS-gel electrophoresis, the hydroxylapaptite/celite eluate showed various protein bands between 25 and 80 kDa. This retinoylated protein was purified 17-fold with respect to the mitochondrial extract.

KEY WORDS: Testes; mitochondria; protein; all-*trans*-retinoic acid; retinoylation; partial purification.

INTRODUCTION

Vitamin A is an essential physiologic regulator of cellular differentiation, the immune response, normal growth, vision, and reproduction. It has generally been believed that most of the functions of vitamin A are mediated by the nuclear retinoic acid receptors, RAR and RXR (Kastner *et al.*, 1994; Mangelsdorf *et al.*, 1994; Wolf, 2000). The activation of retinoic acid (RA) in development and cell differentiation is mediated by these receptors, which interact directly by binding to specific DNA sequences. In addition to binding nuclear retinoid receptors, RA acts elsewhere in the cells (Bolmer and Wolf, 1982; Smith *et al.*, 1989; Varani *et al.*, 1996).

Retinoylation (acylation by RA of protein) is another mechanism by which RA may act on cells. RA is incorporated into proteins of cells in culture (Breitman and Takahashi, 1996; Takahashi and Breitman, 1989, 1990, 1994; Tournier *et al.*, 1996) and into proteins of rat tissues, both in vivo (Myhre *et al.*, 1996) and in vitro (Genchi and Olson, 2001; Myhre *et al.*, 1998; Renstrom and DeLuca, 1989). The retinoylation reaction involves the intermediate formation of retinoyl-CoA (Wada *et al.*, 2001) and subsequent transfer and covalent binding of the retinoyl moiety to protein(s) (Renstrom and DeLuca, 1989). The covalent linkage between RA and protein(s) is a thioester

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Key to abbreviations: BHT, butylated hydroxytoluene; BSA, bovine serum albumin; CoASH, coenzyme A; EDTA, ethylenediaminetetraacetic acid; HTP, hydroxylapaptite; MA, myristic acid; MES, 2-[*N*-morpholino]ethanesulfonic acid; NEM, *N*-ethylmaleimide; PA, palmitic acid; RA, all-*trans*-retinoic acid; RAR, retinoic acid nuclear receptor; RXR, 9-*cis*-retinoic acid specific nuclear receptor; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TRIS, tris(hydroxymethyl)aminomethane.

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bond (Genchi and Olson, 2001; Myhre *et al.*, 1996). The identification of retinoylated proteins is very important to understand the function of retinoylation. Retinoylated proteins that have been identified include cyclic AMP-binding proteins, vimentin, the cytokeratins, and some nuclear proteins (Breitman and Takahashi, 1996; Takahashi and Breitman, 1989, 1990, 1994; Tournier *et al.*, 1996).

Several types of lipid modifications have received considerable attention over the last few years. It has been demonstrated that palmitic, myristic, acetic and phosphoric acids, as well as isoprens farnesol and geranilgeranol, bind to a number of proteins (Schultz *et al.*, 1988; Towler *et al.*, 1988). Retinoylation is one of these covalent modification reactions occurring on proteins. Biochemical similarities exist between retinoylation and acylation (i.e., palmitoylation and myristoylation). In these processes, RA, MA, and PA covalently bind to preformed protein via a thioester bond after the intermediate formation of acyl-CoA and retinoyl-CoA (Renstrom and DeLuca, 1989; Wada *et al.*, 2001). Such covalent modification reactions regulate the interactions of the proteins with cellular membranes, as well as the interactions with other proteins. Physiologically, retinoids, bound to proteins or lipoproteins in the extracellular fluids, are taken up by cell surface receptors, are transferred into the cytoplasm and subsequently bound to intracellular proteins (Blomhoff *et al.*, 1991). Therefore, retinoids do not normally equilibrate in phospholipids of cellular membranes.

It is known that RA is essential for the normal growth of testes (Chaudhary *et al.*, 1989). The steroid biosynthesis is very active into testes mitochondria and moreover is stimulated also by RA; therefore this so active testes mitochondrial process of retinoylation should be bound to steroidogenesis.

MATERIALS AND METHODS

Chemicals

11,12–^{3}H-all-*trans*-Retinoic acid, 50 Ci mmol^{-1} was obtained from Dupont-New England Nuclear; all-*trans*-retinoic acid, 13-*cis*-retinoic acid, *N*-ethylmaleimide, ATP, ADP, GTP, GDP, UTP, CTP, TDP, CoASH, sucrose, TRIS, trichloroacetic acid (TCA), butylated hydroxytoluene, Coomassie Brilliant Blue, EDTA, Pipes, and sodium dodecylsulfate from Sigma-Aldrich (Milano, Italia); hydroxylapatite (Bio-gel HPT) from Bio-Rad; Triton X-100, celite 535, acrylamide, and *N*,*N*′-methylenebisacrylamide from Serva; AMP from Boehringer Mannheim Italia (Milano, Italia); scintillation cocktail from Packard Bioscience (Groningen, The Netherlands). All other reagents used were of the highest purity commercially available.

Preparation of Mitochondria

Sprague-Dawley male rats (200–250 g) were provided with a nutritionally complete rodant diet and water ad libitum. Rats were killed by decapitation and the testes were removed immediately. Rat testes mitochondria were isolated by differential centrifugation as described by Genchi and Olson (2001). Purified mitochondria were suspended in a sucrose/TRIS buffer, pH 7.3, at a concentration of 10–15 mg/mL^{-1}. The purity of the mitochondrial preparation was checked by assaying marker enzymes for lysosomes, peroxisomes, and plasma membranes. This mitochondrial suspension was used immediately or was frozen at -70°C; in both cases the binding activity was the same.

Partial Purification

Rat testes mitochondria were solubilized in 3% Triton X-100 (w/v), 20 mM Na_2SO_4, 1 mM EDTA, and 10 mM Pipes, pH 7.0 (buffer A) at a final concentration of 10 mg protein mL^{-1}. After 15 min at 0°C, the mixture was centrifuged at 15,000 × g for 15 min. The 1.8 mg/200 μL of centrifuged supernatant (Triton extract) were applied to cold hydroxylapatite/celite (5:1) column (Pasteur pipette containing 500 mg of dry material) preequilibrated with buffer A. Elution was performed with 2 mL of buffer A (collecting two fractions of 1 mL each). Then the column was eluted step by step with buffer A in the presence of 50, 100, and 150 mM phosphate, collecting 1 mL of protein solution for each phosphate buffer.

Incubations and Analyses

^{3}H-all-*trans*-Retinoic acid was diluted in ethanol. Mitochondria, Triton extract, and eluted proteins were incubated in the presence of 10 mM ATP, 150 μM CoASH, 27 mM $MgCl_2$, 50 mM sucrose, and 100 mM TRIS, pH 7.4, in a final volume of 0.5 mL at 37°C for 90 min (Genchi and Olson, 2001; Renstrom and DeLuca, 1989). At zero time the reaction was started adding 3–5 μL of the solution of ^{3}H-RA (100 nM, about 400,000 cpm). In the case of pH-dependence, the incubation buffer was 10 mM ATP, 150 μM CoASH, 27 mM $MgCl_2$, 50 mM sucrose, and either 100 mM MES (pH 5.5–6.5) or 100 mM TRIS (pH 7.0–8.5). The reaction was stopped by adding TCA (room temperature) in a final concentration of 5%. The

mixture was centrifuged in an Eppendorf centrifuge at 15,000 × *g* for 10 min. The sediment was extracted seven times with 1 mL $CHCl_3$:CH_3OH (2/1) containing 0.005% of BHT (Bligh and Dyer, 1959). The seventh extract contained <0.1% of the radioactivity added and 1–5% of the amount found in the mitochondrial protein. The pellet was dried and solubilized with 200 μL 1% SDS containing 2 mM EDTA in 40 mM TRIS, pH 7.5 (Genchi and Olson, 2001). This solution, after being suspended in 4 mL of scintillation cocktail, was counted in a TriCarb 1600 TR liquid scintillation counter (Packard). The counting efficiency was about 70%.

Other Methods

Polyacrylamide slab gel electrophoresis of acetone-precipitated samples was performed in the presence of 0.1% SDS according to Laemmli (1970). Samples were solubilized in a solution containing 100 mM TRIS–HCl, pH 6.8, 30 mM dithiothreitol, 10% glycerol, 0.01% Bromophenol Blue, and 2.5% SDS. A mini gel system (8 cm × 10 cm × 1.5 mm) was used. The stacking gel contained 5% acrylamide and the separation gel 12% acrylamide with a ratio acrylamide/bisacrylamide of 30:0.2. Staining was performed by Coomassie Brilliant Blue method. Protein concentration was determined by the Lowry method modified for the presence of Triton (Dulley and Grieve, 1975), using bovine serum albumin as the reference standard.

RESULTS

Properties of Retinoylation Reaction

The retinoylation reaction of rat testes mitochondrial proteins occurred in the presence of ATP, CoASH, and Mg^{++}. The omission of ATP and CoASH in the incubation buffer markedly reduced the extent of retinoylation. Also Mg^{++} was essential for this process; in fact, in Table I it is shown that whether the omission of this cation, or its substitution with Ca^{++} reduced in considerable manner the radioactivity bound to the mitochondrial proteins (8 and 12%, respectively; control value was 21.6 pmoles mg protein^{-1} 90 min^{-1}).

Table I. Effect of Cations on Retinoylation Reaction of Rat Testes Mitochondria[a]

Incubation	Incorporated radioactivity (pmoles mg protein^{-1} 90 min^{-1})	% activity
$+Mg^{++}$	21.6	100
$-Mg^{++}$	1.8	8
$+Ca^{++}$	2.6	12

[a] Retinoylation reaction was carried out under standard conditions and the two substrates were added to incubation buffer at 27 mM concentration at 37°C. The reaction was started at zero time adding 100 nM ^{3}HRA.

Table II. Dependence of Retinoylation Reaction on Nucleotides[a]

Nucleotide	% activity
ATP	100
ADP	47
AMP	18
GTP	30
GDP	19
CTP	17
UTP	15
TDP	12

[a] The different substrates were added to incubation buffer at 10 mM concentration at 37°C. The reaction was started at zero time adding 100 nM ^{3}H-retinoic acid. The control values of the reaction was 19.8 ± 2.1 pmoles mg protein^{-1} 90 min^{-1}. The data are the means of three experiments.

The ATP specificity in retinoylation reaction was investigated in the presence of a variety of nucleotides. The nucleotide concentration was 10 mM. The data reported in Table II shows that retinoylation reaction occurred in the presence of ATP, ADP (about 50% of the control), and GTP (30% of the control). In contrast, retinoylation process did not occur when AMP, GDP or cytosine-, thymidine, and uracil-nucleotides were added to the reaction buffer.

The temperature-dependence of the retinoylation reaction of rat testes mitochondrial proteins is presented in Fig. 1. In an Arrhenius plot a straight line was obtained in the range of 5–37°C. The activation energy as derived from the slope was 43.5 kJ mol^{-1}. The pH effect on the retinoylation reaction is shown in Fig. 2. In the range between 5.5 and 8.5, the optimum pH was found to be 7.5. At pH values lower and highier than 7.5, the retinoylation activity decreased markedly.

Rat testes mitochondria were incubated in the presence of 100 nM ^{3}H-all-*trans*-retinoic acid with increasing concentrations of NEM for 90 min at 37°C. After this incubation time, the radioactivity was measured in the delipidated protein. This cysteine-specific reagent inhibited almost completly (96% at 500 μM) the retinoylation process and the half maximal inhibition was found to be 30 μM (results not shown).

Similarities between retinoylation and acylation (i.e., myristoylation and palmitoylation; Schultz *et al.*, 1988;

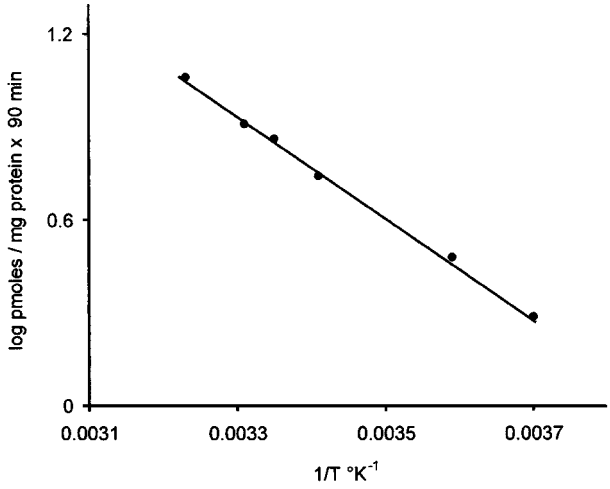

Fig. 1. Temperature-dependence of the retinoylation reaction. Rat testes mitochondria were incubated at the indicated temperatures under the standard conditions for 90 min at 37°C.

Towler *et al.*, 1988) raised the question of whether the same proteins were substrates of these three lipids. Rat testes mitochondria were incubated under standard conditions with ^{3}HRA (100 nM final concentration) in the absence and in the presence of 500 μM myristic and palmitic acids. In these experiments the delipidated proteins had the same binding activity. This result showed that the retinoylation of rat testes mitochondrial protein was not inhibited by high concentrations of MA and PA.

The ATP saturation curve of retinoylation system is shown in Fig. 3. As the concentration of ATP increased, graph of retinoylation activity versus ATP concentration showed sigmoidal behavior. Similar behavior has been obtained with CoASH (not shown). The apparent $K_{0.5}$ for this

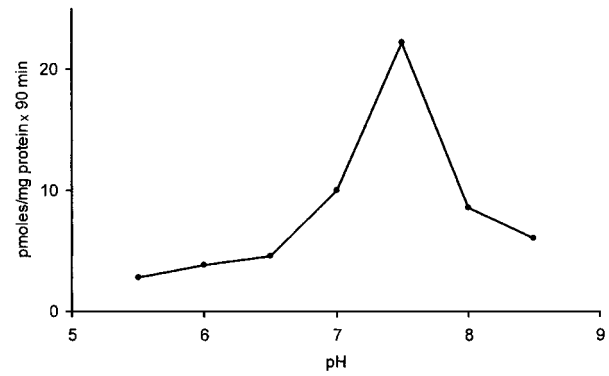

Fig. 2. pH-dependence of the retinoylation reaction. Rat testes mitochondria were incubated at the indicated pH at the conditions in Materials and Methods for 90 min at 37°C.

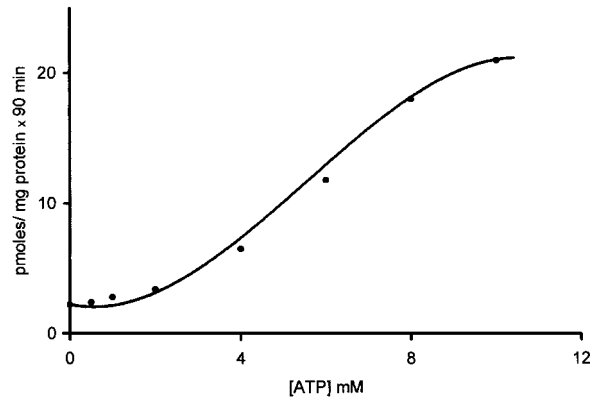

Fig. 3. Relationship between the retinoylation activity on rat testes mitochondrial proteins and ATP concentration. The activity of retinoylation process is plotted as a function of indicated ATP concentrations.

enzymatic system was 6.5 mM ATP and 40.6 μM CoASH, respectively. These sigmoidal graphs for ATP and CoASH indicate that the retinoylation enzymatic system was allosteric with a positive cooperativity.

K_m and V_{max} Values of Retinoylation Reaction and Inhibition by 13-*cis*-Retinoic Acid

To obtain the basic kinetic data of retinoylation reaction on rat testes mitochondrial proteins, the dependence of retinoylation activity on substrate concentration was studied by increasing the concentration of ^{3}H-RA. In three experiments at 37°C an average of 700 $\pm$ 75 nM for the K_m and 100 $\pm$ 18 pmoles mg protein^{-1} 90 min^{-1} for the V_{max} was determined. The inhibition of the retinoylation reaction by 13-*cis*-retinoic acid was analyzed in the presence of different RA concentration. 13-*cis*-Retinoic acid was identified as a competitive inhibitor, since it was found to increase the apparent K_m without changing the V_{max} of the retinoylation reaction. The K_i, which was calculated from the double reciprocal plot of the retinoylation reaction versus substrate concentration, was found to be 1.5 $\pm$ 0.12 μM.

Partial Purification of Retinoylating System From Rat Testes Mitochondria

Rat testes mitochondria were solubilized in Triton X-100 and subjected to chromatography on HTP/celite column (Table III). The column was first eluted with buffer A, and then step by step with buffer A in the presence of 50, 100, and 150 mM phosphate. The first milliliter of the eluate with buffer A had no proteins. The

Table III. Purification of the Retinoylation Proteins From Rat Testes Mitochondria[a]

Purification step	Protein (mg)	Specific activity	Purification (fold)
Extract	9.0	31.6	1.0
HTP/celite	1.2	69.4	2.2
HTP/celite[b]	0.3	537.4	17.0

[a] Incorporation of 100 nM ^{3}H-retinoic acid into proteins incubated for 90 min at 37°C. The binding activity is expressed in pmoles mg protein^{-1} 90 min^{-1} (specific activity).
[b] Eluate with buffer A plus phosphate 150 mM.

proteins eluted from HTP/celite column with buffer A in the presence of 50 or 100 mM phosphate had no binding activities. By this purification steps, eluting the column with buffer A in the presence of 150 mM phosphate, the specific activity of the retinoylating protein (537.4 pmoles mg protein^{-1} 90 min^{-1}) was increased 17-fold with respect to that of Triton extract (69.4 pmoles mg protein^{-1} 90 min^{-1}).

Figure 4 showed the SDS-polyacrylamide gel electrophoresis of mitochondrial extract (lane 2) and Triton-phosphate (150 mM) eluate (lane 3) obtained from rat testes mitochondria solubilized with Triton X-100 and eluted from HTP/celite column. The fraction of lane 3 was substantially purified with respect to mitochondrial extract, although it still contained various protein bands with apparent molecular masses from 25 to 80 kDa.

DISCUSSION

The mechanisms for many actions of retinoic acid were not clearly known until the discovery of retinoic acid receptors (RARs) (Kastner *et al.*, 1995; Wolf, 2000). It has been demonstrated by several investigators that retinoylation of proteins occur in HL-60 cells, in many other cell types and in rat tissue fractions (Breitman and Takahashi, 1996; Genchi and Olson, 2001; Myhre *et al.*, 1996, 1998; Takahashi and Breitman, 1989; Wada *et al.*, 2001). Retinoylation is another mechanism by which RA may act on cells (Pipkin *et al.*, 1991; Takahashi *et al.*, 1991; Takahashi and Breitman, 1989). One metabolic pathway for retinoylation is the formation of retinoyl-CoA

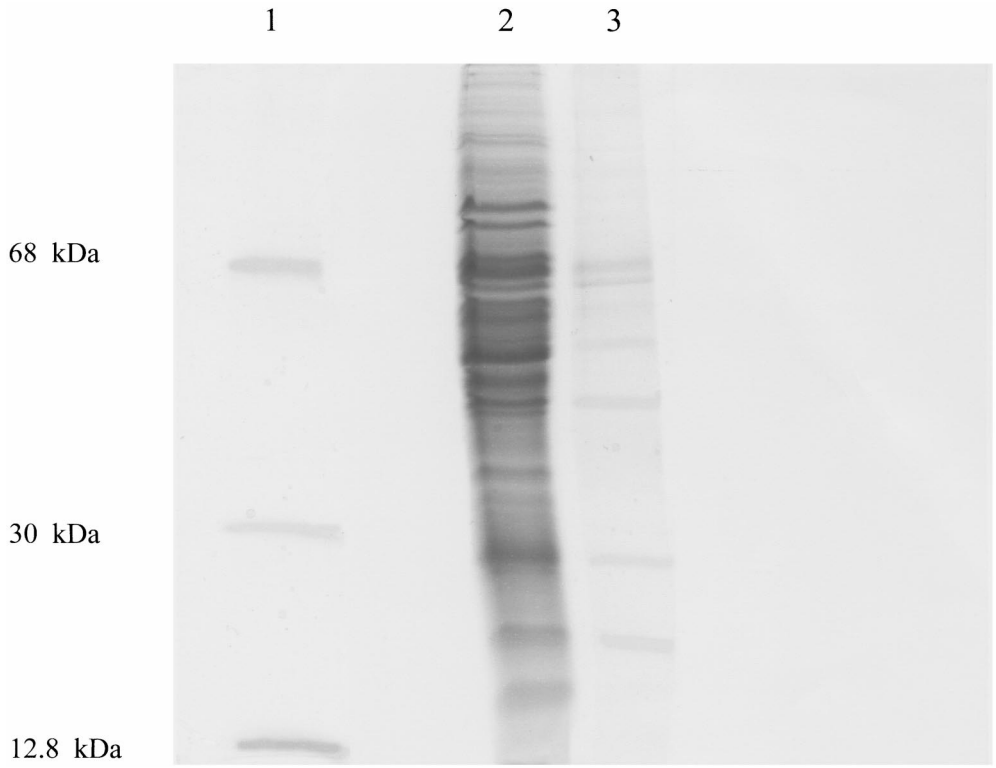

Fig. 4. Partial purification of retinoylating proteins from rat testes mitochondria. SDS gel electrophoresis of fractions obtained during the purification steps. Lane 1 = (from top to bottom) BSA, Carbonic anhydrase, and Cyt C; lane 2 = Triton X-100 mitochondrial extract (310 μg/35 μL); lane 3 = HTP/celite eluate with buffer A plus 150 mM phosphate (10.5 μg/35 μL).

intermediate and subsequent transfer and covalent binding of retinoyl moiety to proteins (Renstrom and DeLuca, 1989).

It was found during the present study that the extent of retinoylation was dependent on the presence of ATP, CoASH, and Mg^{++}. In the absence of these three cofactors the retinoylation was reduced by approximately 90% in rat testes mitochondria. Moreover the retinoylation reaction exhibited substrate specificity (Table I). In fact the reaction occurred only in the presence of ATP, ADP (50% of the control), and GTP (30% of the control). In contrast, in the presence of AMP and pyrimidinic nucleotides triphosphate the reaction did not occur. Also Mg^{++} was found to be essential cation for this process; in fact in the absence of Mg^{++} and in the presence of Ca^{++} the retinoylation process was only 10% of the control.

The retinoylation reaction was temperature-dependent with an activation energy of 44 kJ mol^{-1} (Fig. 1) and pH-dependent showing a marked pH optimum at 7.5, being strongly inactivated at values below 7.0 and above 8.0 (Fig. 2). The binding of *trans*-retinoic acid to the protein(s) of rat testes mitochondria has been studied in the presence of NEM, a reagent with high selectivity for modification of −SH cysteine residues. The *N*-ethylmaleimide reduced strongly the retinoylation process in a concentration-dependent manner with a half maximal inhibition of 30 μM.

The retinoylation reaction on rat testes mitochondria was not affected by high concentrations (500 μM) of myristic and palmitic acids. This was contrary to the results by Wada *et al*. (2001), who showed a high inhibition (90%) of retinoylation in rat liver microsomes with 10 μM long chain fatty acids.

The ATP or CoASH saturation curves of retinoylation process showed sigmoidal behaviors, indicating that this enzymatic process is allosteric, with apparent $K_{0.5}$ of 6.5 mM ATP and 40.6 μM CoASH, respectively.

The K_m and V_{max} values for all-*trans*-retinoic acid were 700 ± 75 nM and 100 ± 18 pmoles mg $protein^{-1}$ 90 min^{-1}, respectively. Besides 13-*cis*-retinoic acid inhibited the retinoylation as a competitive inhibitor with a K_i of 1.5 ± 0.12 μM.

The retinoylating system, eluted with buffer A/150 mM phosphate from HTP/celite column, has been purified 17-fold with respect to the mitochondrial extract (Table II), showing by SDS polyacrylamide gel electrophoresis several protein bands with apparent molecular masses from 25 to 80 kDa (Fig. 4). Among the others, a protein band with a molecular mass of approximately 29 kDa is shown (Fig. 4); this protein should correspond to tritiated protein with the same molecular mass in Fig. 3 of Genchi and Olson (2001).

Testes are very sensitive to vitamin A deficiency. In fact, a vitamin A-deficient diet may cause atrophy of the testes (Wolbach and Howe, 1925). Germ cell development is particularly impaired (Griswold *et al.*, 1989; Sobhon *et al.*, 1979). Spermatidis and spermatocytes, which are sloughed off from the seminiferous tubule soon after vitamin A-deficient rats start to loose weight, become necrotic a few days later (Sobhon *et al*., 1979). In this process, mitochondria become vacuolated, and chromatin forms aggregate along the nuclear envelope. Thus, testes require vitamin A and also possess the binding proteins and the enzymes needed for its metabolism (Eskild *et al.*, 1991).

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