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Evidence for ATP-ase activity of arrestin from bovine photoreceptors

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In vertebrate photoreceptors the soluble protein arrestin (45 kDa) is involved in controlling the light dependent activity of receptor proteins such as transducin or the cGMP-phosphodiesterase. Arrestin has further been identified as the retinal-S-antigen which is assumed to cause the autoimmune disease uveitis. In a first communication a binding of the nucleotide ATP to arrestin was described. In this subsequent study it is shown that arrestin is also able to hydrolyse ATP at a rate of $(5.1 \pm 0.3) \cdot 10^{-3}$ U/mg·min with $C_{\frac{1}{2}} = 93 \pm 5$ nM and a Hill coefficient $n = 1.8 \pm 0.1$ at pH 7.2 and 20°C. These findings suggest a new insight into the process of regulating photoreceptor activity.

Arrestin; ATP-hydrolysis; Rod outer segment; Signal transduction

1. INTRODUCTION

The signal transduction in the vertebrate photoreceptor cell is initiated by light absorption of the photopigment rhodopsin which is located in its outer segment. The light activation is rapidly transferred to channel proteins in the envelope membrane of the outer segment which control the ion currents of the receptor. The transduction process is mediated by a combined action of 4 receptor proteins: a G-protein (transducin) of 79 kDa acts as a diffusible transmitter by giving alternatively and repetitively contact between the light activated meta II rhodopsin and inhibited phosphodiesterase (PDE-I) [1]. During this process the PDE is activated to hydrolyse cyclic guanosine monophosphate (cGMP) which is known to keep the ion channels open [2]. The limitation of the light activity of the receptor is initiated by the action of rhodopsin kinase (68 kDa) which phosphorylates bleached rhodopsin at the C-terminal end and is completed by the binding of the fourth receptor protein, arrestin (45 kDa), to the bleached phosphorylated rhodopsin [3–5].

It is noteworthy that arrestin-like proteins have been detected lately in various other bovine tissues [6], first of all in the pineal gland [7], but beyond that in human medulloblastoma cells as well [8]. Moreover, quite recently another representative of this class of proteins was found in the β-adrenergic receptor and referred to as β-arrestin [9]. Thus it can be assumed that arrestin has a significance beyond the phototransduction process, probably in controlling signal transduction processes in various cells.

As reported earlier by Kühn [10], arrestin activity appears to depend on adenosine triphosphate (ATP). In fact, in a previous paper a binding of the nucleotide ATP to arrestin was described for the first time and also an ADP/ATP exchange was discussed [11]. In particular, it was found that arrestin is capable of binding at least two mols of ATP per mol arrestin and that this binding occurs already at low ATP-concentrations with a half saturation of about 0.1 μM of free ATP.

Furthermore, in flash photometric studies of light absorption and scattering changes, it could be established that arrestin binds already in the meta II state of photoexcited rhodopsin prior to phosphorylation by rhodopsin kinase and that this binding is inhibited by ATP and not ADP [12]. These results suggest a model for arrestin activity in which there is a nucleotide exchange. In such a model the ADP form of arrestin should have a high affinity for photoexcited rhodopsin whereas the ATP form should not. Furthermore, in view of certain homologies in the amino acid sequence between arrestin and transducin [13], an activity of arrestin to hydrolyse nucleotides seems to be quite possible.

In this communication the first direct evidence is given that arrestin is able to hydrolyse nucleotides indeed. Utilizing different, sensitive ATP-detection

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Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; cGMP, cyclic guanosine monophosphate; DTT, dithiothreitol; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PEP, phosphoenolpyruvate; PK, pyruvate kinase; S, arrestin; SDS, sodium dodecylsulfate; TCA, trichloracetic acid; TEMED, *N,N,N',N'*-tetramethylethene diamine; Tris, trishydroxymethyl aminomethane; ros, rod outer segment

methods such as luminescence tests and a γ-P^{32} radio assay, a moderate hydrolysis rate of ATP was detected which showed an allosteric dependence on the substrate concentration.

2. MATERIALS AND METHODS

2.1. *Arrestin purification*

Arrestin was prepared as described by Wilden et al. [14] with some specific modifications to enhance the yield of the active protein.

Bovine eyes were gathered at the slaughter house and kept warm near body temperature until the retinae could be removed 1–2 h after enucleation of the eye bulbs. In this way 50 retinae were isolated from the open eye cup and stored in liquid nitrogen. For the preparation the retinae were thawed in 70 ml 70 mM sodium phosphate buffer (pH 7.2; 2 mM $MgCl_2$/0.1 mM EDTA), shaken vigorously by hand for 1 min, and then stirred gently for 30 min in an ice bath under continuous illumination by the light of an 150 W incandescent lamp (glass filter OG 590/1 mm, Schott, Mainz, Germany). After 30 min centrifugation at 80000 × g (SW-27 rotor of L5-30 ultracentrifuge, Beckman Instr. Inc., Palo Alto, CA, USA) the supernatant was collected and centrifuged again. The final supernatant was stored in aliquots of 2 ml at −20°C. Hereafter this extract is called 'retinae extract'.

For a further purification of arrestin, a normal dark-adapted ros preparation [15] containing 3 mg rhodopsin was homogenized in 2 ml of 70 mM sodium phosphate buffer (pH 7.2/2 mM $MgCl_2$/0.1 mM EDTA/3 mM ATP/1 mM GTP) and illuminated at 20°C by the white light of the 150 W incandescent lamp. After 20 min 2 ml retinae extract was added and the mixture illuminated for another 10 min. The suspension was centrifuged for 20 min at 28000 × g (Biofuge A, rotor 1378, Heraeus Christ, Hanau, Germany). The supernatant was discarded and the pellet washed twice by resuspension in 4 ml of 10 mM HEPES buffer (pH 7.2) followed by a 40 min centrifugation at 28000 × g. The final pellet was resuspended in 2 ml KCl buffer (pH 7.2/2 mM HEPES/500 mM KCl) and incubated in this solution at 4°C in darkness overnight. Thereafter the suspension was centrifuged for 20 min at 28000 × g. The supernatant contained purified arrestin in a concentration range of $(2.9–7.1)\cdot10^{-6}$ M.

2.2. *Protein assay*

A quantitative protein assay was performed by the method described by Bradford [16].

2.3. *SDS-PAGE chromatography*

The purified arrestin was determined by discontinuous SDS-gel electrophoresis described by Laemmli [17] employing slight modifications. The resolving gel was prepared from a 2.5 ml acrylamide stock solution (29.1% acrylamide, 0.9% *N,N'*-methylenebis-acrylamide), 3.4 ml double distilled water, 1.5 ml Tris-HCl (1.875 M, pH 8.8); 75 μl 10% SDS, 4 μl TEMED and 25 μl 10% ammonium peroxidisulfate. The stacking gel was prepared from 0.8 ml acrylamide stock solution, 1.8 ml double distilled water, 0.25 ml Tris-HCl (1.25 M, pH 6.8), 25 μl 10% SDS, 2.5 μl TEMED and 9 μl 10% ammonium-peroxidisulfate. Thus, the resolving gel contains 10%, and the stacking gel 5%, acrylamide.

The gel was prepared in a midget chamber (LKB, Bromma, Sweden) thermostatted at 10°C. The electrode buffer (1.92 M glycine, 0.25 M Tris, 10% SDS) was diluted 1:6 with demineralized water.

The protein samples were prepared by adding 10 μl 10% SDS and 15 μl glycerol to a total of 50 μl. Finally 1 μl of 1 M DTT was added before the sample was vortexed. The operating conditions for the gel electrophoresis were 20 mA at a maximum voltage of 400 V. A high voltage power supply with a volthour integrator (ECPS 3000/150 and VH-1, both Pharmacia, Uppsala, Sweden) was used. The voltage integral for a gel was adjusted to 175 Vh which is about 1 h actual running time.

The gel was stained by the silver staining method as described by Heukeshoven and Dernick [18]. This staining method yields an absolute resolution of 1 ng of protein. Molecular weight markers as carbonanhydrase (29 kDa), egg-albumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase b (97.4 kDa) and β-galactosidase (116 kDa) were obtained from Sigma (St. Louis, MO, USA).

2.4. *Determination of ATP-hydrolysis rate*

ATP-hydrolysis was measured by three different procedures. For the first method a luminescence test is used employing the luciferin-luciferase system as described by Chapelle [19] and Thore [20]. In this case the ATP hydrolysis is followed directly in the sample compartment of the luminometer (direct time dependent measurement). The luminometer is from LKB (Bromma, Sweden), type LKB-1250. The reaction volume consists of 50 μl LKB monitoring reagent, 200 μl sample buffer and 10 μl ATP standard with concentrations varying between 25 and 400 nM. The hydrolysis is started by the rapid addition of 0.5/1.0 μl of the arrestin preparation (3.5/7.1 μM) yielding an enzyme concentration of 7–27 nM. The hydrolysis rate is calculated from the luminometer reading versus time (cf. Fig. 2A). The calibration is readily achieved by the ATP level obtained before the addition of arrestin.

To avoid possible cross reactions between the reagent system and arrestin in the sample the test procedure was modified according to Schmidt and Gräber [21]. Therefore, the ATP hydrolysis is performed separately and stopped at various time intervals so that the remaining ATP can be measured in the luminometer afterwards (time sampling method). For these measurements a total reaction volume of 500 μl is prepared by mixing 250 μl of ATP solution in 20 mM $MgCl_2$ with 250 μl of an arrestin preparation (900 ng protein in 100 mM NaCl, 10 mM Tris-HCl, pH 7.2) so that the initial ATP concentration ranges between 25 and 500 nM and the arrestin concentration is 40 nM for each sample. In the time range of 1–10 min after the start of the hydrolysis samples of 30 μl volume are gathered and rapidly mixed with 30 μl trichloroacetic acid (TCA) for denaturation of the enzyme. From these samples, two times 20 μl are taken and added to the luminometer solution to measure the resting ATP contents as described above. For calibration corresponding samples are prepared from the same ATP standard solution but without addition of arrestin.

As for the third method, a radio label assay was used employing γ-[^{32}P]ATP according to Avron [22] with slight modifications as recently described by Junesch [23]. The samples are prepared with γ-[^{32}P]ATP in the same way as described above for method 2. The sampling intervals are in the range between 1 and 5 min. The radiolabeled samples are further handled as described in [22,23] by forming complexes of $^{32}P_i$-molybdate which are extracted with isobutanol/toluol. The amount of P_i obtained by the ATP-hydrolysis in each sample was measured in a scintillation counter (1600 CA Tri-Carb, Packard Instr. Comp., Meriden, CT, USA) on the P_i-background formed by the intrinsic ATP-degradation.

3. RESULTS

Experiments of ATP hydrolysis were performed in vitro by use of highly purified arrestin. The degree of purification is shown by the SDS-PAGE plots given in Fig. 1. The remaining impurities are less than 0.5‰ w/w.

In a preceding communication [11] we reported a strong binding of ATP to purified arrestin. The binding studies were performed by use of luminescence measurements employing the ATP-indicating luciferin-luciferase enzyme system. Upon repeating these binding studies, it was noticed that following ATP binding

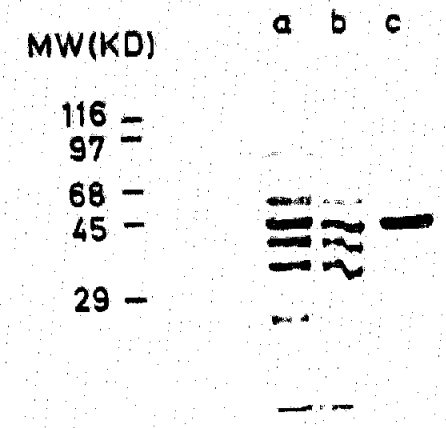

Fig. 1. SDS-PAGE plot of arrestin purification. Lane (a) shows retina extract (1.5 μg protein), lane (b) the first supernatant after centrifugation (1.5 μg protein, see text), lane (c) purified arrestin, in case of highly loaded slot (4 μg protein).

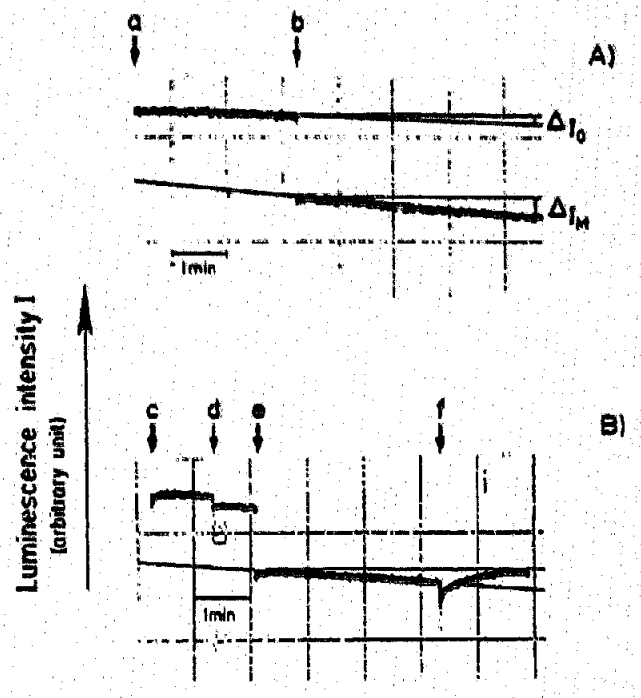

Fig. 2. ATP concentration in dependence of time measured by means of luminescence intensity. (A) (upper trace) luminescence intensity evoked by 40 pmol ATP in the luminometer reaction volume of 250 μl before (a) and after the addition of 4.2 pmol (1 μl) arrestin. Trace (a) shows the small linear decrease of the ATP level due to a system drift and/or the hydrolysis induced by the indicating enzyme system (luciferin-luciferase) itself. $-\Delta I_0$ intensity decrease equivalent to 0.06 pmol ATP in 8 min. Trace (b) gives the initial drop in ATP level by ATP binding [11] followed by an enhanced decrease of ATP level evoked by the addition of a relatively small amount of arrestin (4.2 pmol) yielding an intensity decrease $-\Delta I_m$ equivalent to 0.11 pmol ATP in 4 min. (B) (lower trace) luminescence level evoked by 40 pmol ATP before (c) and after addition of 4.2 pmol denatured arrestin (boiled) (d) to the reaction volume; (e) ATP binding and hydrolysis after addition of 4.2 pmol of native arrestin; (f) 3.5 min after the start of ATP hydrolysis 1 μl phosphoenolpyruvate (100 mM) and 1 μl pyruvate kinase (4.2 mM) are added. After about 2 min the initial ATP level prior to the action of both enzyme systems is re-established.

the luminescence level showed a slow linear decrease that was larger than expected by the indicating enzyme system itself (Fig. 2A). No additional luminescence shift was observed if denatured (boiled) arrestin was applied (Fig. 2B).

In order to prove that ATP is really being hydrolyzed by arrestin, phosphoenolpyruvate (PEP) and pyruvate kinase were added to the test solution. In the example of Fig. 2B this is carried out 3.5 min after the start of ATP hydrolysis by arrestin. After about 2 min the same ATP level is restored to the point that existed prior to the action of both enzyme systems.

In order to verify the preliminary results a comprehensive study of ATP hydrolysis by arrestin was performed by 3 different methods to establish its complete concentration dependence: (1) The direct measurement of ATP hydrolysis: the ATP decrease is followed vs time by the luminescence of the luciferin-luciferase ATP indicating system. (2) The time-sampling method: the ATP hydrolysis is stopped by TCA at a given time interval, and the ATP-turnover is determined by luminescence. (3) A radio assay: the time-sampling method is applied to the hydrolysis of γ-P^{32}-ATP. After the stop of hydrolysis by TCA the ATP turnover is measured by the release of γ-$^{32}P_i$ inorganic phosphate.

The hydrolysis rates obtained with the first method are derived directly from the luminometer data as shown in Fig. 2A.

The results of the third method, i.e. the release of radiolabeled inorganic phosphate (P_i) is plotted in Fig. 3 at various initial ATP concentrations in dependence of time after the start of hydrolysis. For each data point the background level of P_i formed by intrinsic ATP degradation is subtracted. As the turnover in this time range is relatively small compared with the initial ATP concentration, the deviation from a linear rise in P_i seems to indicate a considerable product inhibition of arrestin. For each ATP concentration the hydrolysis rate was derived from the initial slope. According to the limited time resolution these initial rates are certainly smaller than the actual ones.

From the luminescence data obtained with the second method a similar plot (not shown) is derived which gives the residual ATP levels versus hydrolysis time. In this case the initial hydrolysis rates of arrestin could be determined with more accuracy.

The hydrolysis rates obtained by the three methods are plotted in Fig. 4 vs the free initial ATP concentration c_A. The free ATP concentration which could not readily be measured with the methods 2 and 3 was

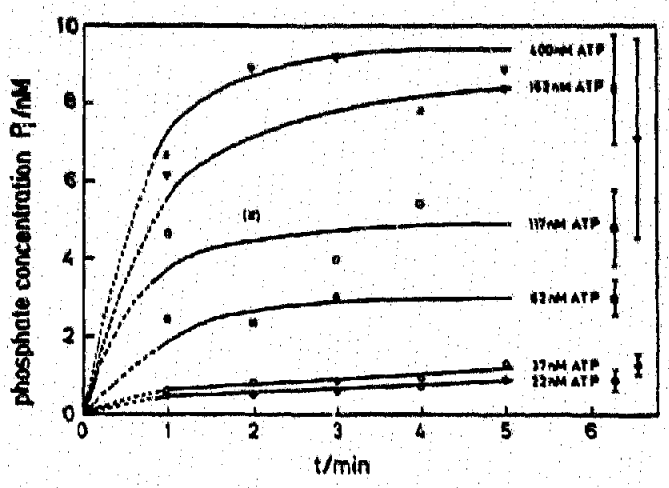

Fig. 3. Arrestin induced ATP hydrolysis as measured by the production of inorganic phosphate versus time. The inorganic radiolabeled phosphate concentration was determined by the release of P^{32} at various time intervals after start of hydrolysis and for various initial concentrations ATP. pH = 7.2, temperature 20°C. The complete diagram is derived from a total of n = 118 samples, about 4 samples per point. The arrestin concentration is 40 ± 2 nM.

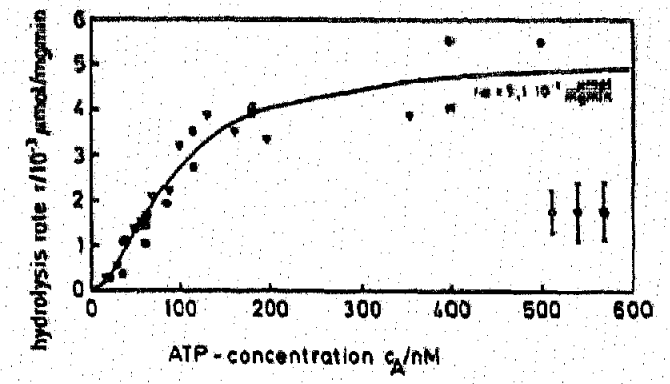

Fig. 4. Initial ATP hydrolysis (r) obtained by arrestin in dependence of the free ATP concentration (c_A). Free ATP concentrations were calculated by use of the ATP binding curve of arrestin given in [11][1]. Open data points (○, △) are average values obtained by luminescence measurement. Total number of single ATP determinations n = 70. Filled data points (■) are derived from γ-P^{32} measurements as plotted vs time in Fig. 3. Total number of single ATP measurements n = 23. The mean curve was obtained by fitting eqn. (1) to luminescence data only (r_{max} = (5.1 ± 2.6)·10⁻³ μmol/mg, $c_{A,1/2}$ = 93 ± 5 nM, n = 1.8 ± 0.1).

determined by use of the known ATP binding curve of arrestin ([11] Fig. 3[1].

The hydrolysis rates given in Fig. 4 show a sigmoidal response with the ATP concentration. The mean curve was obtained by a least square fit of the Hill equation

$$r = r_{max}\frac{c_A^n}{K + c_A^n} \quad (1)$$

according to an overall reaction scheme

$$S + m \cdot A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} SA_m \xrightarrow{k_p} m(A' + P_i) \quad (2)$$

as an approximation valid in case of high cooperativity. ($K = c^n_{A,1/2}$, A = ATP, A' = ADP).

The fit was done with the luminescence measurements only (method 1 and 2) because of their greater statistical significance. As expected, the data from the radiolabel P_i measurements stay slightly below this mean curve.

The appearance of a sigmoidicity ($n > 1$) with the hydrolysis rate data from all 3 different measuring methods shows that the ATP-binding ($m > n > 1$) succeeds cooperatively. The curve fitting procedure results in optimum values for the three parameters: the maximum velocity: r_{max} = (5.1 ± 0.3)·10⁻³ μmol/mg min; the half maximum concentration $c_{A,1/2}$ = 93 ± 5 nM, and the effective number of binding sites n = 1.8 ± 0.1.

From these data it is concluded that at least two ATP molecules bind cooperatively to arrestin. If 75% of the protein is active (cf. [11]) the turnover number for the hydrolysis is calculated from the maximum velocity r_{max} using M_S = 45 kDa to 0.31 ± 0.08 mol ATP/mol arrestin·min or 5.1 ± 0.3 mmol/mol·s.

4. DISCUSSION

The first evidence of membrane bound ATPase activity in rod outer segment was reported by Thacher [24] on the basis of radio labeled ATP measurements. ATPase activity was further communicated by Uhl et al. in 1979 [25] by use of light scattering experiments. After light activation the ATPase appears to pump protons across the disc membrane. The proton translocation, however, effects a volume change in the discs which in turn gives rise to the measured turbidity change. The author described the same phenomenon again in 1989, this time in dependence of various ATPase inhibitors [26,27]. So far there is no further evidence for an ATPase activity in ros apart from these two authors. In both cases, however, the ATPase is supposed to be strongly membrane bound and thus should not be related to the soluble protein arrestin.

The turnover number of the ATP-hydrolysis at 20°C of 5.1 mmol/mol·s is small if compared with the turnover number of the phosphodiesterase PDE with 800 mol/mol·s at 25°C [28] but is well in the same order of magnitude as the GTPase activity of transducin which yields 17 mmol/mol·s at 30°C [29]. The hydrolysis rate shows nearly the same concentration dependence as the ATP binding to arrestin. The $c_{A,1/2}$-value for ATP binding is 97 ± 5 nM[1] which is nearly identical with that for ATP hydrolysis. The difference in the Hill coefficient (2.3 ± 0.1 for binding[1] and 1.8 ± 0.1 for hydrolysis) is not very significant as

[1] The binding data of ATP to arrestin had to be slightly changed after revising the original binding studies reported in [11] into $\Delta c_{A,max}$ = 87 ± 9 nM, n = 2.3 ± 0.1, $c_{A,1/2}$ = 97 ± 5 nM

In both cases the data for high ATP concentration are subject to large deviations. It can be concluded that at least two, probably three, ATP molecules bind cooperatively to arrestin.

The special nucleotide-binding sites of arrestin [30] are not engaged in the complex binding with phosphorylated rhodopsin [1]. Thus, these sites are free to bind ATP. As the concentration for half saturation in ATP binding to arresting is very low (0.1 μM), it is very likely that the complex of arrestin with phosphorylated rhodopsin is saturated by bound ATP.

Following this line of evidence, it is striking to speculate about the still unknown pathway of arrestin action as well in terminating the phototransduction process (a) as in starting regeneration (b):

(a) If the arrestin-rhodopsin complex is broken up by the loss of bound ATP during hydrolysis, then arrestin might be able to pick up ATP again and to form new complexes with other phosphorylated rhodopsin molecules. Furthermore, if the hydrolysis energy is utilized to induce conformational changes in the activated rhodopsin, then the ability of transducin interaction might be irreversibly terminated without any permanent contact with arrestin. That means, at high bleaching rates arrestin should be able to stop the transduction cascade repetitively by inactivating more rhodopsin molecules than free arrestin molecules are available.

(b) The presumed change in rhodopsin conformation induced by the ATP hydrolysis might also be the starting signal for the regeneration process. This is e.g. the dephosphorylation which does not take place while arrestin is bound [31], and very likely also the docking of the retinal binding proteins which facilitate the off-transport of all-*trans* retinal for the reisomerization [32].

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