Stereospecificity of L-*myo*-Inositol-1-phosphate Synthase for Nicotinamide Adenine Dinucleotide†

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ABSTRACT: Partially purified preparations of L-*myo*-inositol-1-phosphate synthase (EC 5.5.1.4) from testis and mammary gland of laboratory rats (*Rattus norvegicus*) were used to show that this enzyme is specific for the *pro-S* hydrogen at C-4 of its cofactor, nicotinamide adenine dinucleotide (NAD). *pro-S* specificity of the first step (reversible oxidation of glucose 6-phosphate to 5-ketoglucose 6-phosphate) was proved by showing that tritium is transferred from [*pro-S*-4-^{3}H]NADH but not from [*pro-R*-4-^{3}H]NADH to glucose 6-phosphate when they are incubated with enzyme. That the stereospecificity in the second oxidation–reduction step (reduction of *myo*-inosose-2 1-phosphate to *myo*-inositol 1-phosphate) is the same as in the first step was shown by demonstrating that tritium from [5-^{3}H]glucose 6-phosphate is incorporated into *myo*-inositol but not into NAD$^+$ and that tritium from [4-^{3}H]NAD$^+$ is not incorporated into *myo*-inositol.

L-*myo*-Inositol-1-phosphate synthase (EC 5.5.1.4), henceforth called synthase in this paper, catalyzes the isomerization of Glc-6-P[1] to 1L-*myo*-inositol 1-phosphate without rearrangement of the carbon chain or the configuration at individual carbons (Eisenberg, 1967, 1978; Chen & Charalampous, 1967; Sherman et al., 1969, 1977; Barnett et al., 1973; Chen & Eisenberg, 1975). It has been purified to homogeneity from rat and bovine testis and well characterized (Maeda & Eisenberg, 1980; Mauck et al., 1980). Naccarato et al. (1974) partially purified it from rat mammary gland.

The postulated mechanism of the action of this synthase (Figure 1) involves oxidation of substrate to 5-ketoglucose 6-phosphate, cyclization to *myo*-inosose-2 1-phosphate, and reduction to 1L-*myo*-inositol 1-phosphate. Step 1 is reversible; Barnett et al. (1973) incubated [4-^{3}H]NADH and cold Glc-6-Pwith rat testis synthase which had been treated with charcoal to remove tightly bound NAD$^+$. Tritium was incorporated into the Glc-6-P at C-5. In another experiment these workers showed that synthase catalyzed the reduction of 5-ketoglucose 6-phosphate by [4-^{3}H]NADH to [5-^{3}H]-glucose 6-phosphate.

Synthase has an absolute requirement for NAD$^+$ (Maeda & Eisenberg, 1980; Mauck et al., 1980); the stereospecificity of transfer of hydrogen in steps 1 and 3 is the subject of the present paper. We designate the positions of the hydrogens at C-4 of NADH as *pro-R* and *pro-S* (Hanson, 1966), corresponding respectively to A and B stereospecificity (You et al, 1978).

Experimental Procedures

Chemicals and Enzymes. NAD$^+$, NADH, Glc-6-P, ATP, aldolase (rabbit muscle, 18 units/mg), glucose-6-phosphate dehydrogenase (yeast, 200 units/mg), lactate dehydrogenase (rabbit muscle, 33 units/mg), and glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle, 90 units/mg) were obtained from Calbiochem; glutamate dehydrogenase and dithiothreitol were from Nutritional Biochemicals; alcohol dehydrogenase (yeast, 3 units/mg) and bacterial alkaline phosphatase (20 units/mg) were from Worthington Biochemical Corp.; [U-^{14}C]glucose, [2-^{3}H]-*myo*-inositol, [1-^{14}C]glucose 6-phosphate, 2,5-diphenyloxazole (PPO), and *p*-bis[2-methyl(5-phenyloxazoyl)]benzene (POPOP) were from New England Nuclear; *myo*-inosose-2 and hexokinase were from Sigma Chemical Co; [5-^{3}H]glucose was from Amersham-Searle. [5-^{3}H]Glucose 6-phosphate was prepared from [5-^{3}H]glucose by the method of Ridley & Kirkwood (1973). [*pro-R*-4-^{3}H]NADH, [*pro-S*-4-^{3}H]NADH, and [4-^{3}H]NAD$^+$ were prepared by the method of Little (1972). The former two were checked for stereospecificity with alcohol dehydrogenase which is specific for *pro-R* hydrogen and glutamate dehydrogenase which is specific for *pro-S* hydrogen (Nelsestuen & Kirkwood, 1971). The synthesized [*pro-R*-4-^{3}H]NADH had 97% of the radioactivity in the *pro-R* position and the [*pro-S*-4-^{3}H]NADH had 90% in the *pro-S* position.

myo-Inositol 2-dehydrogenase (EC 1.1.1.18) was prepared from *Aerobacter aerogenes* by the method of Larner (1962); its activity was 2400 units/mg of protein. Inositol synthase was prepared from testes and mammary glands of rats (Holtzman strain) as described by Byun et al. (1973). The preparations used were purified 69- and 77-fold from KCl extracts of testis and mammary gland, respectively. For some experiments a solution of the enzyme was stirred with Mallinckrodt activated charcoal (100 mg/mL) for 15 min at 0 °C and then centrifuged at 1400*g* for 5 min. The charcoal had previously been washed with 50 mM Tris–acetate buffer, pH 7.4, until washings were free of phosphate (Barnett et al., 1973). All other chemicals employed were of reagent grade and all water was deionized and redistilled from glass.

Analytical Methods. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard. Sugars and *myo*-inositol were determined by the anthrone test (Bartlett, 1959) with glucose as the standard. Solutions were deionized by passage through columns of mixed-bed resins (Amberlite MB3).

Ascending thin-layer chromatography was performed on Eastman 6065 sheets and descending paper chromatography

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[1] Abbreviations used: Glc-6-P, glucose 6-phosphate; NAD$^+$, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; ATP, adenosine 5′-triphosphate; PPO, 2,5-diphenyloxazole; POPOP, *p*-bis[2-methyl(5-phenyloxazoyl)]benzene; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

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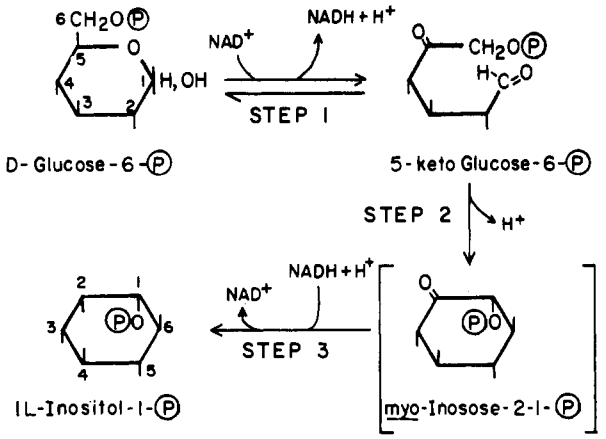

FIGURE 1: Reactions in the synthesis of *myo*-inositol 1-phosphate from glucose 6-phosphate.

on Whatman sheets. The following solvent systems were used: solvent I, 1-butanol/pyridine/water (6:4:3 v/v/v); solvent II, acetone/water (17:3 v/v); solvent III, ethanol/1 M ammonium acetate (3:1 v/v); solvent IV, ethyl acetate/pyridine/water (10:5:4 v/v/v). Tritiated compounds were located on chromatograms by scanning in a Packard radiochromatogram, Model 7201. Sugars were located by staining with alkaline silver nitrate and pyridine nucleotides by fluorescence.

DEAE-cellulose columns (1 × 10 or 1 × 15 cm) previously treated with 0.12 M NH_4HCO_3 and washed with water were used to separate pyridine nucleotides from Glc-6-P and *myo*-inositol 1-phosphate. They were eluted with a NH_4HCO_3 gradient of 0–0.12 M. Pyridine nucleotides were detected in eluted fractions by absorbance at 280 and 340 nm and sugars by thin-layer chromatography and radioactive counts.

Radioactive counting was performed with a Beckman LS-235 liquid scintillation counter. ^{14}C-Labeled compounds were counted in 2 mL of ethanol plus 15 mL of scintillation solution (10 g of PPO plus 0.05 g of POPOP in a mixture of 1 L of ethanol plus 1.5 L of toluene). Tritiated compounds were counted by the method of Bray (1960).

Assay for Inositol Synthase. Radioactivity incorporated into *myo*-inositol 1-phosphate when [1-^{14}C]glucose 6-phosphate and NAD^+ were incubated with the enzyme was determined. The reaction mixture contained, in a volume of 1 mL, 50 mM Tris–acetate buffer, pH 7.4, 6 mM [1-^{14}C]glucose 6-phosphate (2.16×10^6 dpm), 6 mM NAD^+, 5 mM ammonium acetate, and 1.25 mM magnesium acetate. The reaction was started by addition of enzyme, incubated for 2 h at 37 °C, and terminated by heating for 2 min in boiling water. After the solution was cooled to room temperature, alkaline phosphatase (0.2 mL containing 0.4 mg of protein) was added and the solution incubated for 1 h more at 37 °C. Incubation was again terminated by heating for 2 min in boiling water, and the precipitate was removed by centrifugation, washed with 1 mL of water, and recentrifuged. The combined supernatant and washing were deionized by passage through a mixed-bed ion-exchange column. Aliquots of 25–50 μL were chromatographed on a cellulose thin-layer sheet with solvent I. Spots corresponding to *myo*-inositol were scraped from the chromatogram into scintillation vials for counting. Standards of authentic [U-^{14}C]glucose and [2-^3H]-*myo*-inositol were developed on thin-layer chromatograms, the spots were scraped off, and their radioactivities were counted in the same manner as for solutions being assayed for synthase activity. Ten replications averaged recoveries of 70% for [U-^{14}C]glucose and 50% for [2-^3H]-*myo*-inositol. Activities of enzyme preparations, in terms of radioactivity transferred to *myo*-inositol, were 470 (testis) and 50 (mammary gland) dpm per mg of protein per min.

Table I: Incorporation of Radioactivity from Specific [^3H]NADH into Glucose and *myo*-Inositol When Incubated with Inositol Synthase for 2 h[a]

		treatment of enzyme		radioactivity[e] (%) in	
	enzyme[b]	charcoal[c]	heat[d]	glucose	*myo*-inositol
[*pro-R*-4-^3H]NADH	M	–	–	1.5	1.1
		–	+	1.3	*f*
[*pro-R*-4-^3H]NADH	T	–	–	0.6	0.2
		–	+	0.2	*f*
		+	–	0.8	0.3
		+	+	0.14	*f*
[*pro-S*-4-^3H]NADH	M	–	–	6.5	0.18
		–	+	0.14	*f*
[*pro-S*-4-^3H]NADH	T	–	–	7.6	0.36
		–	+	0.3	*f*
		+	–	7.7	0.2
		+	+	0.03	*f*

[a] Incubation mixtures (1 mL) had the following concentrations: 50 mM Tris–acetate, pH 7.4, 6 mM [1-^{14}C]glucose 6-phosphate (2.16×10^6 dpm), 6 mM NAD^+, 5 mM ammonium acetate except as noted in footnote *c* below, 1.25 mM magnesium acetate, and 1.5–1.6 mg of enzyme preparation. [b] M = mammary; T = testis. [c] 0.05 μmol of NAD^+ was added to the reaction mixture when charcoal-treated enzyme was used, and NH_4^+, concentration 0.19 M [as $(NH_4)_2SO_4$], was added when enzyme that had not been treated with charcoal was used. [d] Heated in boiling water for 2 min. [e] Percentage of radioactivity of reaction mixture. [f] Not done.

Results and Discussion

Stereospecificity of Step 1. The stereospecificity of the first step was determined by incubating (for 2 h) reaction mixtures like those of the assay, except that [*pro-R*-4-^3H]- or [*pro-S*-4-^3H]NADH was substituted for the NAD^+ and cold Glc-6-P for the [1-^{14}C]Glc-6-P used in the assay. Treatment with phosphatase and chromatographic separation of glucose and *myo*-inositol were performed as in the assay method. Little radioactivity was incorporated into Glc-6-P from [*pro-R*-4-^3H]NADH, but the mammary and testis synthases catalyzed transfer of 6.5 and 7.6%, respectively, of the radioactivity of [*pro-S*-4-^3H]NADH to Glc-6-P (Table I).

To locate the tritium, we isolated glucose and *myo*-inositol from reaction mixtures containing [*pro-S*-4-^3H]NADH by chromatography on Whatman 3MM paper (solvent II). Upon degradation of the glucose (3.16×10^5 dpm) by the method of Bevill et al. (1965), glycerol (C-4–6) with 1.52×10^5 dpm was isolated on Whatman 3MM paper (solvent IV). Oxidation of the glycerol with $NaIO_4$ yielded formate (C-5), isolated as ammonium formate, having 8.78×10^4 dpm or 27.8% of the radioactivity of the original glucose. No radioactivity was observable in the dimedone derivative of the formaldehyde from C-4 and C-6. The isolated *myo*-inositol (1×10^5 dpm) was incubated with *myo*-inositol 2-dehydrogenase and NAD^+. From one portion of the incubated solution *myo*-inosose-2 was separated by thin-layer chromatography with solvent II, after deionization on a mixed-bed (MB3) column. It was found to be devoid of radioactivity. Another portion of the incubated solution was streaked on duplicate Whatman 3MM papers and developed in solvent III for 36 h. One chromatogram was scanned for radioactivity, and on the other, pyridine nucleotides were detected by fluorescence and sugars by alkaline silver nitrate staining. Most of the radioactivity was in the pyridine nucleotides; a small amount was in unreacted *myo*-inositol,

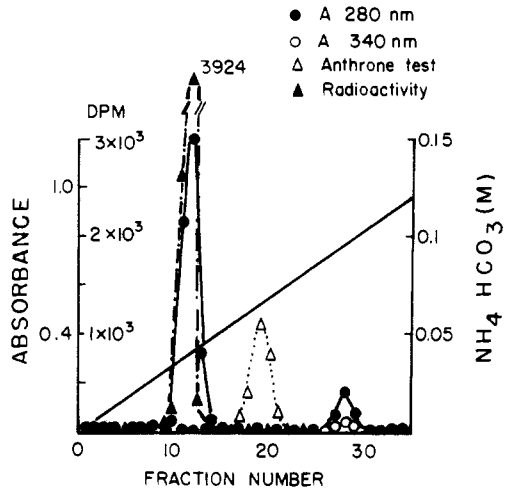

FIGURE 2: DEAE-cellulose chromatography with a NH_4HCO_3 gradient of reaction mixture in which cold glucose 6-phosphate and [4-^{3}H]NAD$^+$ were incubated with rat testis inositol synthase (details in text).

but none was in *myo*-inosose-2.

The transfer of tritium from [*pro-S*-4-^{3}H]NADH to Glc-6-P when they were incubated with the enzyme confirms the reversibility of step 1 as reported by Barnett et al. (1973). This step is specific for the *pro-S* hydrogen. The degradation experiments showed that the tritium was attached to C-5 of glucose 6-phosphate and C-2 of *myo*-inositol 1-phosphate.

Stereospecificity of Step 3. Two experiments were done to determine ifthe specificity of step 3 is the same as that of step 1. In both, the proportions of reactants were the same as in the standard assay procedure except that the NH_4^+ concentration was 0.19 M. Incubation was continued for 4 h.

In the first experiment [4-^{3}H]NAD$^+$ (6.6×10^5 dpm) and cold Glc-6-P were used. The incubation mixture, without phosphatase treatment, was fractionated on a DEAE-cellulose column with a gradient of NH_4HCO_3. Figure 2 shows all of the radioactivity in the pyridine nucleotides; no exchange of tritium had occurred between the [4-^{3}H]NAD$^+$ and the Glc-6-P.

In the other experiment [5-^{3}H]glucose 6-phosphate (2.84×10^7 dpm) was incubated with enzyme and cold NAD$^+$. One batch, not treated with phosphatase, was fractionated on the DEAE-cellulose column. Figure 3 shows that only Glc-6-P and *myo*-inositol 1-phosphate contained tritium. No tritium was transferred to NAD$^+$ or NADH in the synthesis of *myo*-inositol 1-phosphate. A second batch of reaction mixture was treated with phosphatase, deionized with a monobed resin, and chromatographed on a cellulose thin-layer sheet with solvent I. Twenty-two percent of the original radioactivity was recovered from the *myo*-inositol spot.

The results reported in Figures 2 and 3 are consistent with a mechanism in which both steps 1 and 3 have the same specificity; tritium appears in *myo*-inositol but not in NAD$^+$ when [5-^{3}H]Glc-6-P is the substrate, and it does not appear in *myo*-inositol when labeled NAD$^+$ is in the reaction mixture. If steps 1 and 3 had opposite stereospecificity tritium from either [4-^{3}H]NAD$^+$ or [5-^{3}H]Glc-6-P would appear in *myo*-inositol. Thus, since the enzyme has *pro-S* specificity in step 1, it must have *pro-S* specificity in step 3 as well. A more direct way to have determined the stereospecificity of the third step would have been to incubate *myo*-inosose-2 1-phosphate with synthase and stereospecifically labeled NADH. Unfortunately, *myo*-inosose-2 1-phosphate was not available to us

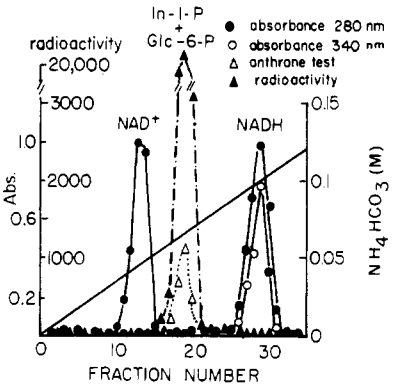

FIGURE 3: DEAE-cellulose chromatography with a NH_4HCO_3 gradient of reaction mixture in which [5-^{3}H]glucose 6-phosphate and cold NAD$^+$ were incubated with rat testis inositol synthase (details in text).

at the time of these experiments, and our attempts to synthesize it failed.

Several other enzymatically catalyzed reactions have been described in which two sequential pyridine nucleotide linked hydrogen transfers are involved. Of these, UDPglucose dehydrogenase (EC 1.1.1.22) (Schiller et al., 1972) and UDPglucose 4-epimerase (EC 5.1.3.2) (Nelsestuen & Kirkwood, 1971) are NAD linked and specific for the *pro-S* hydrogen in both steps. On the other hand, β-hydroxy-β-methylglutaryl-CoA reductase (EC 1.1.1.34) is NADP linked and specific for the *pro-R* hydrogen in both steps (Dungan & Porter, 1971).

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Specificity of Heme Oxygenase: A Study with Synthetic Hemins†

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ABSTRACT: A large number of synthetic iron porphyrins were enzymatically oxidized by a microsomal heme oxygenase preparation from rat liver. They all had in common two vicinal propionic acid residues at C_6 and C_7. Iron porphyrins of type I were not substrates of the enzyme. Iron porphyrins that carried electron-withdrawing substituents (acyl residues) at C_2 and C_4 were substrates of heme oxygenase, although the product yields were reduced. Several iron porphyrins, such as hemin XIII (**4**) and hemin III (**5**), were better substrates of heme oxygenase than the natural substrate hemin IX (**1**). The enzymatic oxidation was selective for the α-methine bridge, and the α-biliverdins obtained were reduced by Biliverdin reductase to the corresponding α-bilirubins. Preincubation of the enzymatic system with hemin IX (**1**) and hemin XIII (**4**) in the absence of NADPH resulted in an inhibition of their oxidation. The iron-free porphyrins which carried two vicinal propionic acid residues at C_6 and C_7 were also found to be inhibitors of the enzymatic system when preincubated with the latter. The presence of hematochemin IX (**18**) suppressed the enzymatic oxidation of hemin IX (**1**).

Heme IX (**1**) (Figure 1), the prosthetic group of hemoglobin and of a large number of hemoproteins, is one of the most abundant compounds of the general metabolism. It is degraded by a heme oxygenase which oxidizes the α-methine bridge of heme IX with the formation of biliverdin IX-α (**2**) (Tenhunen et al., 1969). Heme oxygenase is a membrane-bound enzyme, and it has been recently solubilized and purified (Yoshida & Kikuchi, 1979; Maines et al., 1977).

The enzymatic reduction of biliverdin IX-α (**2**) by a biliverdin reductase gives rise in mammals to bilirubin IX-α (**3**), which is the overwhelming bulk of biliary bilirubin (Figure 2). The enzymatic oxidation of the α-methine bridge is highly specific, although some minor traces of the other bilirubin isomers (IX-β, IX-γ, and IX-δ) have been detected (Blumenthal et al., 1977). The enzymatic oxidation of **1** poses two questions, the first with regard to the substrate specificity of heme oxygenase and the second with regard to the mechanism which leads to the specific oxidation of the α-methine bridge. We have recently shown (Frydman et al., 1979) that heme oxygenase oxidizes not only heme IX but also several isomeric hemins and that the oxidation was specific for the α-methin bridge. We therefore prepared by synthesis a series of iron porphyrins to make a systematic study of both problems, substrate specificity and methine bridge selective oxidation.

The question of the selective oxidation at the α-methine bridge has been extensively discussed (O'Carra, 1975). It stands in striking contrast with the chemical oxidation of heme IX which gives rise to all four possible isomers of biliverdin IX (Bonnett & McDonagh, 1973). In the chemical oxidation, the biliverdin IX-α is formed in a slight excess over the other isomers. It has been proposed that the enzymatic α specificity arises as a result of intrinsic electronic features of the heme IX molecule itself (Lemberg, 1956; Pullman & Perault, 1959), which would favor a preferential attack at the α bridge. A second interesting proposal was that the α specificity is a result of the interaction of heme with the heme binding sites in the different hemoproteins in such a manner that only the α bridge is oxidized (O'Carra, 1975). Support for this proposal was found by carrying out chemical oxidations of anomalous hemoglobins which resulted in biliverdin IX isomers of various types (Brown & Docherty, 1978). The third proposal suggested that the active site of heme oxygenase imposes the α specificity of the cleavage (Yoshida & Kikuchi, 1978). Our preliminary work (Frydman et al., 1979) indicated that hemins XIII (**4**) and III (**5**) (Figure 3), which are isomers of hemin IX (**1**) (Figure 1), were substrates of heme oxygenase. Both **4** and **5** carried the two vicinal propionic acid residues at C_6 and C_7. The present study was therefore performed by using synthetic iron porphyrins which carried two vicinal propionic acid residues at C_6 and C_7 (Figures 1 and 3). The iron porphyrins **6** and **7** (Figure 3), which lacked this feature, were also prepared and assayed as substrates. The iron 2,4-diacetyl-, 2,4-dipentanoyl-, 2,4-diisopentanoyl-, and 2,4-dilauroyldeuteroporphyrins IX (**8**, **9**, **10**, and **11**, respectively), which carried electron-withdrawing substituents, enabled us to examine the possible electronic effects on the substrate of heme oxygenase.

The enzymatic oxidations of these hemins were carried out with a rat liver microsomal heme oxygenase which was induced by cobaltous chloride. The use of the synthetic iron porphyrins also allowed us to examine the specificity of biliverdin reductase on the different biliverdins formed during the oxidation of the former.

Materials and Methods

Materials. Hemin IX and bilirubin IX-α were purchased from Sigma Chemical Co. and were purified before use. Protoporphyrin XIII (**4**) (iron-free porphyrins will have the same numbering as their iron dervatives), protoporphyrin III

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