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PURIFICATION AND PROPERTIES OF THE MAJOR ISOZYMIC FORM OF CYTOPLASMIC GLYCEROL-3-PHOSPHATE DEHYDROGENASE FROM RABBIT LIVER

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Summary

The major isozymic form of *sn*-glycerol-3-phosphate dehydrogenase (*sn*-glycerol-3-phosphate:NAD^+ 2-oxidoreductase, EC 1.1.1.8) has been purified from rabbit liver using a simplified three-step chromatographic procedure involving an ion exchange and two affinity chromatography steps. The 1200-fold purified enzyme is electrophoretically homogeneous, nucleotide-free, and possesses a specific activity of 295 units/mg and an isoelectric point of 6.5. A steady-state kinetic analysis was applied to both the forward and reverse reactions. The NADH oxidation reaction was found to adhere to Michaelis-Menten behavior with K_m values of 22 μM and 75 μM for NADH and dihydroxyacetone phosphate, respectively. In the NAD reduction reaction, sigmoidal kinetic patterns were observed when NAD was the variable substrate whereas with *sn*-glycerol-3-phosphate as the variable substrate, strictly hyperbolic kinetics were observed. The apparent K_m values for NAD and glycerol-3-phosphate were 83 and 909 μM, respectively. By comparison with published reports, these results demonstrate that the rabbit muscle and liver isozymes of *sn*-glycerol-3-phosphate dehydrogenase have different kinetic properties and suggest that the liver isozyme is better adapted to participation in glyconeogenesis in vivo.

The enzyme glycerol-3-phosphate dehydrogenase (*sn*-glycerol-3-phosphate: NAD^+ 2-oxidoreductase, EC 1.1.1.8) plays a diverse set of metabolic roles in carbohydrate and lipid metabolism. The enzyme catalyzes the reduction of dihydroxyacetone phosphate to produce *sn*-glycerol-3-phosphate (glycerol-3-*P*), which provides the carbon backbone for membrane and storage lipids [1]. The

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Abbreviation: glycerol-3-*P*, *sn*-glycerol 3-phosphate.

enzyme may also help to maintain the redox state during anaerobic glycolysis [2] and may allow for the shuttle of reducing equivalents into the mitochondrion [3—5].

The multiple physiological roles of glycerol-3-*P* dehydrogenase are reflected in its structural complexity. The enzyme has been shown to exist in several isozymic forms. Multiple forms of the enzyme have also been observed in chickens [6], rats [7—10], *Drosophila* [11], pigs [12] and humans [13]. The isozymes of glycerol-3-*P* dehydrogenase from rabbit tissues are immunologically distinct [14]. Lee and Craine have shown [15] that a partially purified preparation of the enzyme from rabbit liver has kinetic properties which are significantly different from those of the well-characterized muscle form. The amino acid compositions of the rabbit liver and muscle form are reportedly different [16]. Ostro and Fondy [17] have recently demonstrated that the major isozymes from liver and muscle are identical by some physical-chemical criteria.

This communication reports on a simplified procedure for the bulk isolation of the major isozymic form (with isoelectric point of 6.5) of glycerol-3-*P* dehydrogenase from rabbit liver. In addition, we describe some of the physical and kinetic properties of this isozyme.

Materials and Methods

All solutions were prepared from distilled, deionized, and charcoal-treated water with reagent grade chemicals of the highest quality available. All buffer solutions contained 1 mM EDTA and 1 mM mercaptoethanol unless otherwise stated. Enzyme grade ultrapure ammonium sulfate was obtained from Schwarz/Mann (Orangeburg, N.Y.). All biochemicals were obtained from Sigma Chemical Company (St. Louis, MO). Enzyme solutions were prepared fresh daily by passing the enzyme, which was stored as a suspension in ammonium sulfate, through a Bio-Gel P-30 column (Bio-Rad Laboratories, Richmond, CA).

Enzyme specific activity was determined spectrophotometrically by observing the rate of oxidation of NADH at 340 nm. Enzyme samples were added to 1 ml 50 mM triethanolamine buffer (pH 7.5)/0.1 mM NADH/0.3 mM dihydroxyacetone phosphate. A Beckman model 25 spectrophotometer with a constant-temperature cell at 30° C was used for all assays. Protein concentrations were determined by the method of Warburg and Christian [18]. 1 unit of glycerol-3-*P* dehydrogenase is defined as the amount of enzyme required to oxidize 1 μmol NADH per min.

Steady-state kinetics were conducted using a Farrand Model Mk-1 fluorescence spectrophotometer equipped with an 8-position turret cell holder maintained at 30° C. The excitation and emission wavelengths were 340 and 460 nm, respectively, and the slits were kept at 5 nm for the excitation and 10 nm for the emission monochromator. The kinetic analysis was carried out using the computer program [19] which fitted the data to Eqn. 1.

$$\frac{v}{E} = \frac{V[A][B]}{K_{ia}K_B + K_A[B] + K_B[A] + [A][B]} \quad (1)$$

From replicate runs the kinetic constants were found to be reproducible to within 20%.

DEAE-cellulose was prepared by washing 10 g material with 500 ml 0.25 M Na_3PO_4/0.025 N NaOH, washing twice with 500 ml distilled water and then 500 ml 0.5 M H_3PO_4 followed by copious amounts of water. The cellulose was then equilibrated against the eluting buffer. The trinitrobenzene affinity gel was prepared by the method of Kornbluth et al. [20] and 6-phosphogluconate affinity gel was prepared as described by McGinnis and deVillis [21]. These affinity materials were suspended in water and stored at 4° C.

Polyacrylamide electrophoresis was performed as described by Davis [22] with ammonium persulfate as the initiator and using 7.3% crosslinked acrylamide. The electrophoresis was performed in a Bio-Rad Model 150 gel electrophoresis cell using pH 8.5 buffer (0.6 g Tris, 2.88 g glycine per l) and 4 mA per gel. SDS gel electrophoresis was performed using 0.1% SDS in the samples and buffer [23]. Isoelectric focusing gels were prepared and focused as described by the ampholyte supplier (Bio-Rad). The focusing was performed at a constant potential of 200 V and each run was stopped after 18 h. The pH gradient was determined using a Bio-Rad Pro-pHiler and miniature pH electrodes. All gels were stained for total protein by using 0.1% Coomassie blue in 7% acetic acid and were destained in 10% acetic acid in a Bio-Rad diffusion destainer. Gels were stained for enzyme activity by the method of Fondy et al. [10]. Gel scans were obtained on a Beckman Model 25 spectrophotometer using a gel scanner accessory at 650 nm for the activity stain or 600 nm for the protein stain.

Enzyme purification

All steps in the enzyme purification were performed at 0—4° C. Rabbit livers were removed from freshly-killed New Zealand does and homogenized with 2 vols. 50 mM triethanolamine buffer (pH 7.5). The pH was readjusted to 7.5 with NH_4OH and the homogenate stirred for 1 h at 4° C. The homogenate was then filtered through several layers of cheesecloth and centrifuged for 30 min at 12 000 rev./min in a Beckman model J-21B centrifuge using a model JA-14 centrifuge head. The supernatant was brought to 60% saturation with $(NH_4)_2SO_4$, stirred overnight and then centrifuged at 12 000 rev./min for 30 min. The precipitate was dissolved in sufficient 50 mM triethanolamine buffer to bring the volume to twice that of the original tissue homogenate. The solution was applied equally to 6 separate 1.4 × 7 cm columns of trinitrobenzene affinity gel and flow rate of 0.5—1.0 ml/min was maintained. After the sample was applied, each column was washed with 100 ml buffer and the enzyme was eluted with 0.1 mM NADH.

The combined active fractions from trinitrobenzene affinity chromatography were concentrated to approx. 10 ml and then applied to a 0.9 × 9 cm 6-phosphogluconate affinity column equilibrated with 50 mM triethanolamine buffer (pH 7.5). After washing with buffer, the enzyme was eluted with 0.2 M DL-glycerol-3-phosphate (flow rate 0.5 ml/min).

The combined enzymically active fractions were equilibrated with 5 mM phosphate buffer (pH 8.2)/approx. 10 μM NADH to help prevent loss of enzyme activity. The sample was then concentrated to 5 ml and applied to a 0.9 × 28 cm DEAE-cellulose column equilibrated with the same buffer. Elution

was effected by a 0—0.1 M NaCl salt gradient. The active fractions were combined, concentrated to approx. 5 ml, dialyzed against 60% saturation $(NH_4)_2SO_4$/50 mM triethanolamine (pH 7.5) and stored at 4°C.

Results

Purification of liver glycerol-3-P dehydrogenase. The enzyme purification scheme is summarized in Table I. The initial step of the purification, $(NH_4)_2SO_4$ fractionation, could be omitted without affecting the results, although this step facilitated the flow and increased the capacity of the first affinity chromatography column. The trinitrobenzene-agarose chromatography resulted in the removal of most of the extraneous protein with an 80-fold purification of the enzyme. This step was accompanied by approx. 80% loss of enzymic activity although higher yields were occasionally obtained. The yields were found to be dependent on the amount of time the enzyme was in contact with the affinity matrix. The enzyme was found to elute as a single, sharp peak immediately following the addition of a 0.1 mM NADH solution.

The combined active fractions from the trinitrobenzene-agarose column were further purified approx. 3-fold with a 93% yield using the 6-phosphogluconate affinity chromatography column. Enzyme eluted very sharply with 0.2 M DL-glycerol-3-*P* (pH 7.5). Electrophoresis of the combined fractions showed three major protein bands and a single enzymically active band corresponding to the center protein band (Fig. 1a).

The enzyme activity was generally obtained in approx. 100% yield (or in some cases, greater than 100%) from the DEAE-cellulose column. After this final step in the purification, the protein migrated as a single band in electrophoresis with a mobility identical to the band which stained for enzymic activity (Fig. 1b). The overall purification factor was approx. 1200 and the enzyme obtained had a specific activity of nearly 300 units/mg (Table I). A single band was obtained when a sample of the purified enzyme was subjected to SDS electrophoresis after heating the enzyme to 100°C in the presence of SDS, to dissociate the subunits.

TABLE I

PURIFICATION OF RABBIT LIVER GLYCEROL-3-PHOSPHATE DEHYDROGENASE

Based on the purification from a single fresh rabbit liver weighing 90 g.

Fraction	Total units	Total protein (mg)	Specific activity (units/mg)	% Recovery	Purification Purification (-fold)
Soluble homogenate	4120	17 200	0.24	100	1
0—60% $(NH_4)_2SO_4$ salt fraction	3500	10 084	0.35	85	1.46
AgTNB *	678	23.8	28.6	16	119
AgPGA **	644	6.9	93	15	388
DEAE-cellulose ***	766	2.6	295	18	1229

* Agarose-trinitrobenzene affinity column, enzyme eluted with 0.1 mM NADH.

** Agarose-6-phosphogluconic acid affinity column, enzyme eluted with 0.2 M DL-Glycerol-3-P.

*** Equilibrated with 5 mM phosphate buffer, pH = 8.2; eluted with NaCl salt gradient.

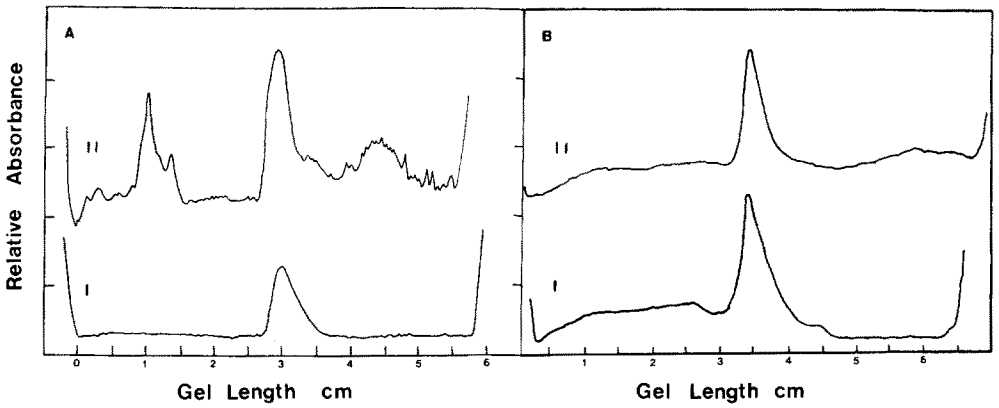

Fig. 1. A. Spectrophotometric scan of an electrophoresis gel obtained on the enzyme fraction from the agarose-6-phosphogluconate column. Scan I was stained for enzyme activity and scan II was stained for protein. B. Spectrophotometric scan of an electrophoresis gel obtained on the enzyme after the complete purification process. Scan I was stained for enzyme activity and scan II was stained for protein.

Further criteria of purity were provided by isoelectric focusing and spectral analysis of the enzyme. Isoelectric focusing of the enzyme (Fig. 2) showed a single well-defined activity peak corresponding to an isoelectric point of 6.5. An ultraviolet spectrum of the purified glycerol-3-*P* dehydrogenase exhibited a maximum absorption at 277 nm and has a 280 nm/260 nm absorption ratio equal to 1.45 ± 0.05.

Kinetics of glycerol-3-P dehydrogenase. The purified enzyme was subjected to steady-state kinetic analysis for both the forward (dihydroxyacetone phosphate reduction) and reverse (glycerol-3-*P* oxidation) reactions at pH 7.5. For each substrate or coenzyme, velocity measurements were made at several concentrations of the variable substrate with all other concentrations constant. For the forward reaction, the velocity exhibited a hyperbolic dependence on substrate and coenzyme concentrations. A least-squares fitting procedure was used to calculate the K_m values of 22 μM and 75 μM for NADH and dihydroxyacetone

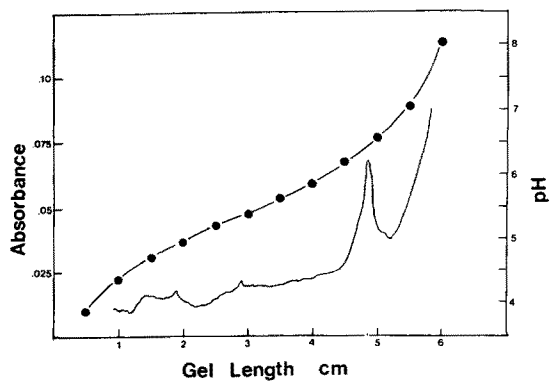

Fig. 2. Isoelectric focusing of *sn*-glycerol-3-*P* dehydrogenase. The purified enzyme was focused in a 5.8 cm gel. The solid trace corresponds to an absorbance scan obtained after staining for enzymic activity. The points correspond to pH measurements on the gel.

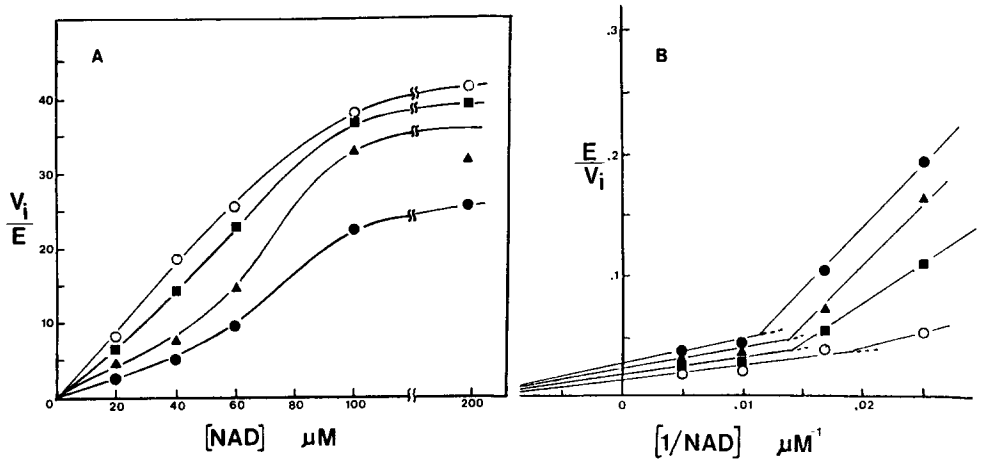

Fig. 3. Effect of substrate concentrations on the initial velocity of the reverse reaction. A: initial velocity v. NAD concentration at the following constant concentrations of *sn*-glycerol-3-*P*: ●, 0.2 μM; ▲ 0.4 μM; ●, 0.8 μM; ○, 1.0 μM. B: double reciprocal plots of the date presented in A.

phosphate, respectively. For the reverse reaction, the kinetic patterns are more complex. Although the velocity depends hyperbolically on the concentration of glycerol-3-*P*, the dependence of velocity on NAD concentration was sigmoidal at the lowest concentrations of glycerol-3-*P*. The K_m value for glycerol-3-*P* is 909 μM and the corresponding apparent K_m for NAD is 83 μM as established from the data shown in Fig. 3.

Discussion

The enzyme, glycerol-3-*P* dehydrogenase, was purified 1200-fold from rabbit liver homogenate. The greatest purification was achieved by the affinity chromatography steps [20,21] included in the procedure. The sequential use of these 2 affinity columns yields a solution of glycerol-3-*P* dehydrogenase containing 2 major protein contaminants which are removable by DEAE-cellulose chromatography. The enzyme obtained after this last step is homogeneous as judged by polyacrylamide gel electrophoresis, isoelectric focusing and by electrophoresis in the presence of SDS.

Although glycerol-3-*P* dehydrogenase is readily purified from skeletal muscles [9,24—26], the lower quantities of enzyme present in liver have made the purification from this latter tissue much more difficult. Otto et al. [16] have used a lengthy procedure to prepare electrophoretically homogeneous enzyme and Ostro and Fondy [17] have recently reported the purification of glycerol-3-*P* dehydrogenase from a number of rabbit tissues including muscle, liver, mammary gland and heart. Using preparative isoelectric focusing they were able to obtain the major liver isozyme focusing at pH 6.5 as well as a minor from focusing at pH 6.3. The simplified procedure reported here is designed to rapidly yield only the major isozyme.

van Eys et al. [27] reported that the absorption spectrum of glycerol-3-*P* dehydrogenase, when crystallized from rabbit muscle, is indicative of the presence of a non-protein component which strongly absorbs light of 260 nm.

Otto et al. [16] observed a similar phenomenon with the rabbit liver enzyme and they report A_{280nm}/A_{260nm} ratios of 1.05 and 1.5 for nucleotide-bound and nucleotide-free enzymes, respectively. The enzyme purified by the procedure reported here has a A_{280nm}/A_{260nm} ratio of 1.45 and, therefore, is nucleotide-free by this criterion.

The question of the similarities and differences between the rabbit muscle and liver enzymes has recently been addressed by Ostro and Fondy. [17]. They have isolated the two major isozymes from these tissues and have shown that they are identical with respect to several physical and catalytic properties. However others have reported differences in amino acid compositions between the two forms [16]. The two enzymes also appear to differ in their kinetic behavior under some conditions in that the liver enzyme shows homotropic interactions with NAD as the substrate whereas the muscle enzyme strictly follows Michaelis-Menten behavior [28,29]. Apparently the difference in catalytic properties of the two enzymes are a reflection of subtle structural differences which are not observable by most techniques. A similar situation also exists for other isozymes. For example the slight differences in amino acid composition of chicken muscle and liver glycerol-3-*P* dehydrogenase are associated with significantly different catalytic and functional properties [6]. Multiple isozymes and subfractions have also been observed with alcohol dehydrogenase and a number of explanations for their existence have been put forward including deamination [30], sulfhydryl group oxidation [31], and different conformational states [32]. Further studies will be required to elucidate the structural and functional interrelationships of the multiple forms of glycerol-3-*P* dehydrogenase.

The first kinetic study of rabbit liver glycerol-3-*P* dehydrogenase was carried out using partially purified enzyme [15]. We have reinvestigated some of the kinetic properties using highly purified enzyme and a sensitive fluorometric assay. Although Lee and Craine [15] reported a sigmoidal dependence of of velocity on glycerol-3-*P* concentration, whereas hyperbolic kinetics were found here, we do concur in the finding of a sigmoidal dependence on NAD concentration.

Otto et al. [16] studied the kinetics of rabbit liver glycerol-3-*P* dehydrogenase in phosphate buffer at pH 7.6 and, in general the kinetic constants obtained are similar to those found here with the striking exception of the K_m for dihydroxyacetone phosphate which is nearly 10 times the value found in this study. Part of this apparent discrepancy may be explained by the fact that phosphate is a noncompetitive inhibitor of the liver enzyme with respect to dihydroxyacetone phosphate [33]. The sigmoidal kinetic patterns were not found by Otto et al. [16] with NAD as the variable substrate. However, the limited concentration ranges employed by these workers would not have allowed the observation of the type of sigmoidal kinetics observed with our enzyme preparation.

Potentially, glycerol-3-*P* dehydrogenase can serve in various metabolic functions. Recent studies of enzyme activities in chicken livers under various dietary conditions [34] and in rat hepatomas [35] suggest that the liver isozyme has a primary role in gluconeogenensis from lipid-derived glycerol. The homotropic interactions of NAD with the liver enzyme support this conclusion

of an important role for the enzyme in glycerol-3-*P* oxidation. This idea is further supported by the lower apparent K_m value for NAD with the liver isozyme compared with that from skeletal muscle [16].

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References

1 Kennedy, E.P. (1957) Annu. Rev. Biochem. 26, 119—148
2 Denten, R.M., Yorke, R.E. and Randle, P.J. (1966) Biochem. J. 100, 407—419
3 Ringler, R.L. and Singer, T.P. (1958) Arch. Biochem. Biophys. 77, 229—232
4 Ringler, R.L. and Singer, T.P. (1959) J. Biol. Chem. 234, 2211—2217
5 Estabrook, R.W. and Sacktor, R. (1958) J. Biol. Chem. 233, 1014—1019
6 White, H.B. and Kaplan, N.O. (1969) J. Biol. Chem. 244, 6031—6039
7 Ross, C.R., Curry, S., Schwartz, A.W. and Fondy, T.P. (1971) Arch. Biochem. Biophys. Acta 145, 591—603
8 Fondy, T.P., Solomon, J. and Ross, C.R. (1971) Arch. Biochem. Biophys. 145, 604—611
9 Fondy, T.P., Ross, C.R. and Sollohub, S.J. (1969) J. Biol. Chem. 244, 1631—1644
10 Fondy, T.P., Herwig, K.J., Sollohub, S.J. and Rutherford, D.B. (1971) Arch. Biochem. Biophys. 145, 583—590
11 O'Brien, S.J. and MacIntyre (1972) Biochem. Genet. 7, 141—161
12 Behnke, J.N. (1973) Ph.D. dissertation, Michigan State University
13 Lehmann, F.G. and Pfleiderer, G. (1968) Hoppe-Seyler's Z. Physiol. Chem. 349, 1777—1785
14 Lee, Y.P. and Choy, P.C.C. (1974) J. Biol. Chem. 249, 476—481
15 Lee, Y.P. and Craine, J.E. (1971) J. Biol. Chem. 246, 7616—7622
16 Otto, J., Raggi, A., Machleidt, W. and Bucher, T. (1972) Hoppe-Seyler's Z. Physiol. Chem. 353, 332—344
17 Ostro, M. and Fondy, T.P. (1977) J. Biol. Chem. 252, 5575—5583
18 Warburg, O. and Christian, W. (1942) Biochem. Z. 310, 384—421
19 Cleland, W.W. (1963) Nature 198, 463—465
20 Kornbluth, R.A., Ostro, M.J., Rittman, L.S. and Fondy, T.P. (1974) FEBS Lett. 39, 190—194
21 McGinnis, J.F. and deVellis, J. (1974) Biochem. Biophys. Res. Commun. 60, 186—195
22 Davis, B.J. (1965) Ann. N.Y. Acad. Sci. 121, 404—427
23 Nakos, G. and Martenson, L. (1971) Biochem. 10, 455—458
24 Baranowski, T. (1949) J. Biol. Chem. 180, 535—541
25 Bentley, P., Dickinson, F.M. and Jones, I.G. (1973) Biochem. J. 135, 853—859
26 Van Eys, J., Judd, J., Ford, J. and Womack, W.B. (1964) Biochemistry 3, 1755—1762
27 Van Eys, J., Nuenke, J. and Patternson, Jr., M.K. (1959) J. Biol. Chem. 234, 2308—2313
28 Black, W.J. (1966) Can. J. Biochem. 44, 1301—1317
29 Bentley, P. and Dickinson, F.M. (1974) Biochem. J. 143, 19—27
30 Theorell, H. (1970) in Pyridine Nucleotide-Dependent Dehydrogenases (Sund, H., ed.), pp. 121—126, Springer-Verlag, Berlin
31 Jornvall, H. (1973) Biochem. Biophys. Res. Commun. 53, 1096—1099
32 Lutstorf, U.M., and von Wartburg, J.P. (1969) FEBS Lett. 5, 202—205
33 Lee, Y.P., Craine, J.E. and Lee, A.S.T. (1973) J. Biol. Chem. 248, 5610—5615
34 Harding, Jr., J.W., Pyeritz, E.A., Copeland, E.S. and White, H.B. (1975) Biochem. J. 146, 223—229
35 Harding, Jr., J.W., Pyeritz, E.A., Morris, H.P. and White, H.B. (1975) Biochem. J. 148, 545—550