Evidence for Direct Hydrogen Transfer During Glyceraldehyde-3-Phosphate Dehydrogenase Catalysis*

W. S. Allison,** M. J. Connors, and D. J. Parker+

From the Graduate Department

of Biochemistry, Brandeis University

Waltham, Massachusetts 02154

(Publication No. 621)

Received January 16, 1969

Summary - An irreversible one-turnover reaction between 1-^{3}H-glyceraldehyde-3-phosphate and the lobster muscle glyceraldehyde-3-phosphate dehydrogenase (TPD) coenzyme complex has been carried out. Analysis of the products of this reaction has shown that DPN^{3}H is formed and tritium is not incorporated into the enzyme. These results suggest that hydrogen is transferred directly from the reduced substrate to the coenzyme during TPD catalysis.

It has been reported that tritium is specifically incorporated into tryptophan residues of yeast alcohol dehydrogenase (1.), rabbit M_4 lactic dehydrogenase (2.), and pig heart malic dehydrogenase (3.) when the enzymes are equilibrated with their respective ^{3}H-substrates in the presence of

---

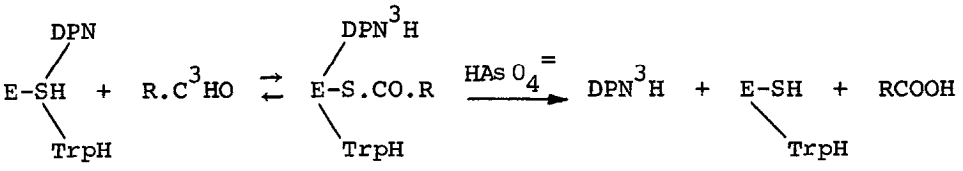
* Supported by Research Grant GM-14,481 of the National Institutes of Health.

** Research Career Development Awardee K3-GM-12,638 of the National Institutes of Health.

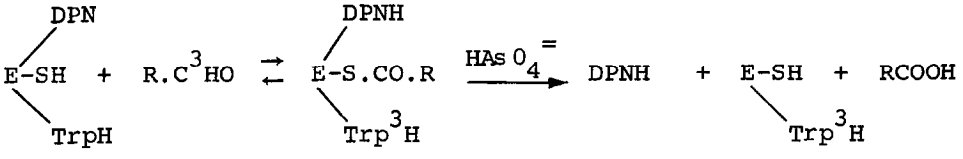
+ Postdoctoral Trainee of the National Institutes of Health Training Grant T01 GM-0212. Present address, Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio.

DPN under alkaline conditions. On the basis of these findings and others (4.), it has been suggested that hydrogen transfer is mediated through the alternate reduction and reoxidation of indolenine intermediates during catalysis by these dehydrogenases (3.). It is possible to carry out an irreversible one-turnover hydrogen transfer reaction when glyceraldehyde-3-phosphate (GAP) is added to the enzyme-DPN complex of glyceraldehyde-3-phosphate dehydrogenase (TPD) in the presence of arsenate. Analysis of the products of such a one-turnover reaction carried out with 1-^{3}H-GAP should distinguish between direct hydrogen transfer mechanism (eq. 1) or a tryptophan mediated hydrogen transfer mechanism (eq. 2).

1.)



2.)



Materials: Lobster TPD was prepared as described previously (5.). DPN^{3}H was prepared as described by Schellenberg (6.) and was purified by ion exchange chromatography on DEAE-Sephadex (HCO_3^- form) before use. 1-^{3}H-GAP was prepared enzymatically and was freed from DPN and DPNH as shown in Fig. 1. Immediately before use in the one-turnover experiments the maximally

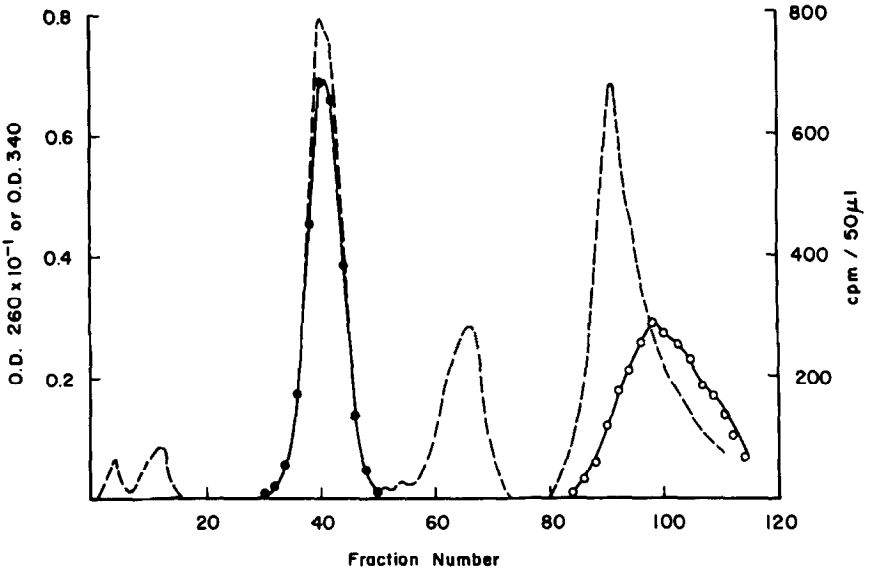


Fig. 1. Purification of 1-^{3}H-GAP - A reaction mixture which contained: 3-phosphoglyceric acid, 150 mg; ATP, 30 mg; DPN^{3}H, 10 µmoles; 0.5 M $MgSO_4$, 0.1 ml; 3-phosphoglyceric phosphokinase, 0.2 mg; and TPD, 1 mg in 3 ml of 0.05 M NH_4HCO_3 was incubated for 5 min at room temperature at which time DPNH oxidation ceased. It was applied to a 1.5 x 20 cm column of DEAE-Sephadex which was equilibrated with 0.01 M NH_4HCO_3. The column was eluted with a linear bicarbonate gradient with the use of mixing chamber containing 400 ml of 0.01 M NH_4HCO_3 and a reservoir chamber containing 400 ml of 0.7 M NH_4HCO_3. 4 ml fractions were collected. The optical density was determined at 280 mµ (●) and 340 mµ (o). The radioactivity of 50 µl samples was also determined (- - - -).

---

activated lobster TPD-DPN complex was prepared as follows. 1% solutions of the enzymes in 5 mM EDTA, pH 7.0 were treated with Norite A to remove bound DPN. After treatment with charcoal the DPN complexes were reconstituted by the addition of a twofold excess of 98% DPN and dithiothreitol to a final concentration of 0.01 M. The excess DPN and the dithiothreitol were removed from the reconstituted enzyme by gel filtration through Sephadex-G-25 equilibrated with 5 mM EDTA, pH 7.0.

The E280/260 ratio of the reconstituted enzyme is 1.03 and reduces 50 μmoles of DPN per mg per min under the assay conditions described previously (5.).

Results: The one-turnover reaction - 2 μmoles (55,000 cpm) of 1-^{3}H-GAP was added to a reaction mixture which contained 60 mg (1.7 μmoles of enzyme subunit) of the reconstituted lobster TPD-DPN complex and 100 μmoles Na_2HAsO_4 in 18 ml of 0.05 M NH_4HCO_3. Coenzyme reduction ceased after 12 min at which time 1.1 μmole of DPN was formed as determined spectrophotometrically at 340 mμ. Solid urea was then added to the reaction mixture to a final concentration of 8 M. This solution was dialyzed against 300 ml of 0.01 M NH_4HCO_3 which was changed twice at 6 hr intervals.

Fractionation of the dialysate - After the addition of carrier DPN and DPNH the combined dialysates were subjected to ion exchange chromatography to separate unreacted 1-^{3}H-GAP from DPNH as shown in Fig. 2. Fractions 18-36 contained DPN. Fractions 47-58 contained 1-^{3}H-GAP (10,000 cpm) which had not reacted. The DPNH was recovered in fractions 80-104 and contained 8000 cpm of ^{3}H. Therefore, DPN^{3}H has been qualitatively identified as a reaction product of the one-turnover reaction.

Analysis of the protein for tritium incorporation - After 18 hrs of dialysis the protein fraction contained 15,000 cpm of ^{3}H. To reduce protein sulfhydryl groups which may have been oxidized during urea denaturation and dialysis, this solution was incubated with 0.01 M dithiothreitol at room temperature

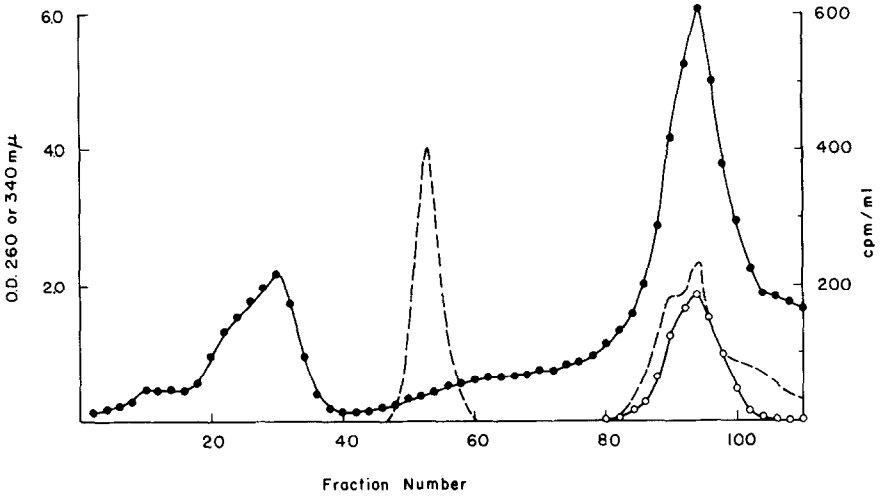


Fig. 2. Fractionation of the dialyzed reaction products. The dialyzed reaction products (see text) to which 5 mg of DPN and 10 mg of DPNH were added were applied to a 1.5 x 20 cm column of DEAE-Sephadex which was equilibrated with 0.01 M NH_4HCO_3. After application of the sample the column was washed with 300 ml of 0.01 M NH_4HCO_3 and then eluted with the linear gradient described in the legend to Fig. 1. The optical density was determined at 260 mμ (●) and 340 mμ (o). Radioactivity was also determined (---).

for 2 hrs in the presence of 8 M urea. Then iodoacetate was added to a final concentration of 0.05 M and the incubation was continued for 2 hrs. The protein solution was then dialyzed exhaustively against 10^{-3} M HCl. After this dialysis the protein solution contained 8000 cpm of ^3H.

After reduction and carboxymethylation the protein was digested with 1 mg of trypsin in 0.5% NH_4HCO_3 for 5 hrs. at 37°. The digest was freeze-dried dissolved in 5 ml of 0.1 M NH_4OH, and subjected to gel filtration on Sephadex G-25 as shown in Fig. 3. The three tryptophan residues in the lobster TPD subunit are in positions 83, 192, and 309 (7.). The tryptic pep-

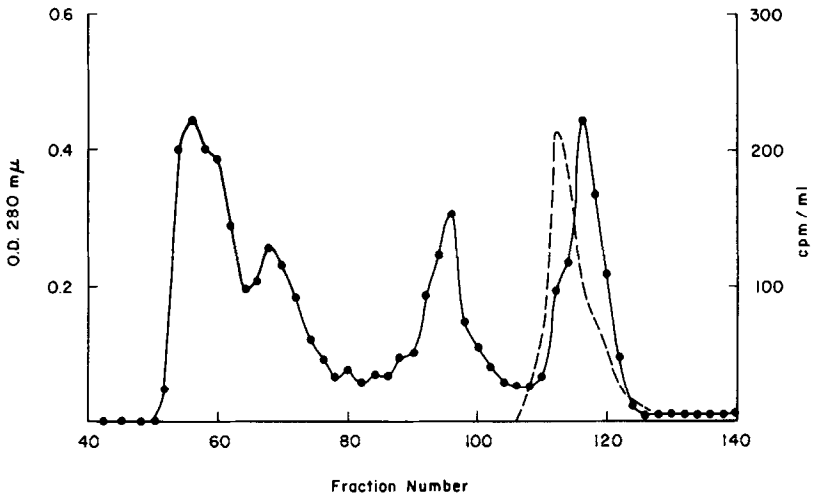


Fig. 3. Gel filtration of the tryptic digest on Sephadex G-25. The tryptic digest of the reduced, carboxymethylated lobster TPD was applied to a 3.0 x 100 cm column of Sephadex G-25 which was equilibrated and eluted with 0.05 M NH_4OH. The optical density at 280 mμ (●) and the radioactivity (---) of the 5 ml collected fractions was determined.

---

tides which contain trp-83 and trp-309 are not retained by Sephadex G-25 and appear in fractions 52-65. Since these fractions are not radioactive, ^3H is not incorporated into trp-83 and trp-309. The tryptic peptide which contains trp-192 is asp.trp.arg. This peptide characteristically appears in fractions 110-125 when tryptic digests of lobster TPD are fractionated with this particular G-25 column. Although the radioactive material and u.v. absorbing material in these fractions are not superimposed, fractions 110-125 were combined and freeze-dried.

The freeze-dried material in fractions 110-125 which contained 6000 cpm of ^3H was fractionated by ion exchange chromatography on DEAE-Sephadex as shown in Fig. 4. The 280 mμ absorbing material in fractions 47-58 was combined and freeze-dried. This

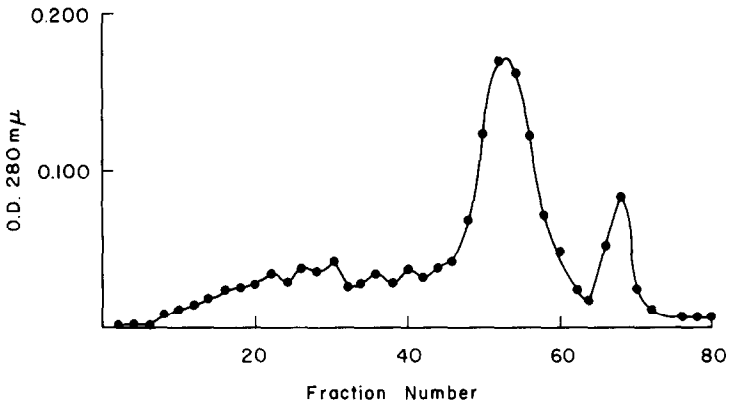


Fig. 4. Purification of asp.trp.arg on DEAE Sephadex - Fractions 110-125 from the Sephadex G-25 column (Fig. 3.) were combined, freeze dried, and applied to a 1.5 x 20 cm column of DEAE-Sephadex which was equilibrated with 0.05 M NH_4-acetate, pH 8.0. The column was washed with 200 ml of 0.05 M NH_4-acetate pH 8.0 and then eluted with an acetate gradient with the use of a mixing chamber containing 500 ml of 0.05 M NH_4-acetate, pH 8.0 and a reservoir chamber containing 0.2 M NH_4-acetate, pH 6.0. The radioactivity of 0.5 ml samples of the 5 ml collected fractions was not above background.

material was not radioactive. When submitted to quantitative amino acid analysis, an acid hydrolysate of this material was shown to contain aspartic acid and arginine in an equimolar ratio and traces of tryptophan. Analysis of the freeze-dried material by paper electrophoresis showed that it contained a single peptide which was neutral at pH 6.5 and basic at pH 3.5. After electrophoresis at both pH's the peptide gave a strong color reaction with the Ehrlich reagent showing that the peptide was indeed asp.trp.arg.

The DEAE-Sephadex column was eluted with 0.1 M acetic acid. This failed to remove the radioactivity which was applied to the column. On the basis of the charge characteristics of the small tryptic peptides of lobster TPD, it is concluded that the

radioactivity applied to the column represents decomposition products of either ^{3}H-GAP or DPN^{3}H or both which dialyze slowly.

Discussion: The analysis of the products of the one-turnover experiment clearly indicate that hydrogen is transferred directly from the 1-position of GAP to the coenzyme during TPD catalysis at neutral pH. Therefore, the hydrogen transfer reaction catalyzed by TPD appears to differ from that of other dehydrogenase (1,2,3). TPD catalysis differs from that of alcohol dehydrogenase, lactic dehydrogenase, and malic dehydrogenase in two respects. First, a thiol ester intermediate is formed during TPD catalysis and secondly TPD is specific for the 4B-position of DPNH (8.). These differences, especially the first, may be responsible for the apparently different mechanisms of hydrogen transfer catalyzed by these dehydrogenases.

References:

1.) Schellenberg, K. A., J. Biol. Chem. 240, 1165 (1965).
2.) Schellenberg, K. A., J. Biol. Chem. 242, 1815 (1967).
3.) Chan, T. L., and Schellenberg, K. A., J. Biol. Chem. 243, 6284 (1968).
4.) Schellenberg, K. A., and McLean, G. W., J. Am. Chem. Soc. 88, 1077 (1966).
5.) Allison, W. S., and Kaplan, N. O., J. Biol. Chen. 239, 2140 (1964).
6.) Schellenberg, K. A., J. Biol. Chem., 241, 2446 (1966).
7.) Davidson, B. E., Sajgo, M., Noller, H. F., and Harris, J. I., Nature 216, 1181 (1967).
8.) Levy, H. R., and Vennesland, B., J. Biol. Chem. 238, 85 (1957).