*DIPHOSPHOPYRIDINE NUCLEOTIDE: A COFACTOR FOR THE POLYNUCLEOTIDE-JOINING ENZYME FROM ESCHERICHIA COLI**

By Baldomero M. Olivera† and I. R. Lehman

DEPARTMENT OF BIOCHEMISTRY, STANFORD UNIVERSITY SCHOOL OF MEDICINE, PALO ALTO, CALIFORNIA

Communicated by Arthur Kornberg, April 27, 1967

The search for enzymes responsible for the joining of DNA molecules has led to the identification and partial purification of an enzyme from *Escherichia coli* which links preformed polynucleotides through phosphodiester bonds.[1] The reaction requires that the polynucleotides to be joined be part of a double-stranded structure, and that a divalent cation and a "cofactor" be added The cofactor, present in extracts of *E. coli*, was found to be heat-stable, adsorbable to Norit, and could not be replaced by ATP or any other known nucleoside triphosphate. We have now purified the cofactor extensively and found it to be identical with diphosphopyridine nucleotide (DPN). The present report provides evidence for this identification.

Materials and Methods.—The preparation of substrates as well as the assay and purification of the polynucleotide-joining enzyme have been described in the previous paper.[1] Assay of cofactor activity was performed using a reaction mixture identical to that used for assay of the polynucleotide-joining enzyme except that an excess of enzyme (0.1 unit) and a level of cofactor producing approximately half maximal velocity were added.[2] One unit of cofactor activity is the amount which promotes the transformation of 1 $\mu\mu$mole of P^{32} 5′-phosphoryl terminus of polydeoxythymidylate (dT) to a form insusceptible to bacterial alkaline phosphatase in 1 minute under these conditions.

DPN, reduced DPN (DPNH), triphosphopyridine nucleotide (TPN) (>95% pure by enzymatic assay), and rabbit muscle lactic dehydrogenase[3] were obtained from the Sigma Chemical Co. DPN analogues (3-pyridine aldehyde-, thionicotinamide-, deamino-, and 3-acetyl pyridine deamino-DPN[4]) were purchased from Mann Research Laboratories. Diadenosine 5′-5′ diphosphate (AppA) was the generous gift of Dr. John Moffatt of the Syntex Institute of Molecular Biology. Venom phosphodiesterase[5] and *N. crassa* DPNase[6] were purchased from Worthington Biochemical Co. Potato nucleotide pyrophosphatase was purified according to Kornberg and Pricer.[7] Diethylaminoethyl Sephadex (DEAE-Sephadex) and Sephadex G-10 were obtained from Pharmacia.

Ultraviolet spectra were measured with a Cary recording spectrophotometer, model 14. Radioactive samples were counted using a Nuclear-Chicago model 186 gas-flow counter equipped with a micromil window.

Results and Discussion.—Purification of cofactor: An extract (70 ml) of *E. coli* (strain 1100, generously provided by Professor H. Hoffman-Berling) was prepared as described previously[1] except that the cells were suspended in 0.01 *M* Tris-HCl buffer pH 8.0 containing 0.005 *M* β-mercaptoethanol and 0.001 *M* ethylenediaminetetraacetate (EDTA). The extract was brought to pH 4.5 by the addition of 1 *N* HCl and the precipitated material was removed by centrifugation. The super-

natant solution, neutralized by the addition of 0.5 N KOH, was applied to a column of DEAE-Sephadex A-25 (12 × 2.2 cm) equilibrated with 0.02 M Tris-HCl, pH 8.0. Active material was eluted during the first half of a linear gradient (1,500 ml total volume) from 0.02 to 0.2 M Tris-HCl, pH 8.0. The pooled DEAE-Sephadex fractions were concentrated 20-fold by evaporation under reduced pressure, then filtered through a column of Sephadex G-10 (22 × 1 cm) equilibrated with 0.01 M Tris-HCl, pH 8.0. Cofactor activity appeared in the void volume and was separated from the bulk of the ultraviolet-absorbing material which was retarded by the gel. A summary of the purification is given in Table 1.

Identification of purified cofactor as DPN: The absorption spectrum at pH 2, 7, or 12 of the Sephadex G-10 fraction was indistinguishable from that of authentic DPN or TPN (commercially available preparations purified from yeast). Treatment of this fraction with L-lactate and the DPN-specific muscle lactic dehydrogenase[3] produced a new absorption band with a maximum at 340 mμ (Fig. 1). The A_{340}/A_{260} ratio of the enzymatically treated Sephadex G-10 fraction was identical to that of known DPNH formed from DPN in the presence of lactate and lactic dehydrogenase, implying that DPN was the only component of the Sephadex G-10 fraction contributing to the 260-mμ absorption.

Authentic DPN was then tested for cofactor activity and found to satisfy completely the cofactor requirement of the reaction. The specific activity of authentic DPN (107 units/A_{260}) was the same as that of the Sephadex G-10 fraction (108 units/A_{260}). Thus, the cofactor activity of the Sephadex G-10 fraction could be quantitatively accounted for by its DPN content. To ensure that these assays were in fact measuring the linkage of dT segments, the product of a reaction in which DPN served as cofactor was sedimented in an alkaline sucrose gradient. Polydeoxythymidylate chains were observed with molecular weights as much as 16-fold greater than the dT substrate, a result identical to that obtained when a boiled extract of *E. coli* was used as cofactor.

The cofactor activity of the Sephadex G-10 fraction responded to a variety of enzymes in a manner expected of DPN. Thus, incubation of cofactor (0.2 ml, A_{260} = 1.4) for 30 minutes with venom diesterase (10 units[8]) or potato nucleotide pyrophosphatase (42 units), both of which hydrolyze the pyrophosphate bond of DPN, or *N. crassa* DPNase (60 units), which attacks the nicotinamide-ribose linkage, resulted in complete loss of activity (<1% remaining). As noted earlier,[1] the cofactor was unaffected by treatment with bacterial alkaline phosphatase. More definitively, reaction of the Sephadex G-10 fraction with L-lactate and lactic dehydrogenase and the subsequent appearance of the characteristic DPNH spectrum (Fig. 1) resulted in a decrease in cofactor activity which was equivalent to that found upon conversion of known DPN to DPNH (Table 2).

TABLE 1

PURIFICATION OF COFACTOR

Fraction	Total activity	Specific activity (units/A_{260})*
Extract	25,650	1.1
Acid fraction	15,000	11.0
Concentrated DEAE-Sephadex	11,270	66.8
Sephadex G-10	6,000	108.0

* Cofactor units per milliliter of solution with an absorbance of 1.0 at 260 mμ.

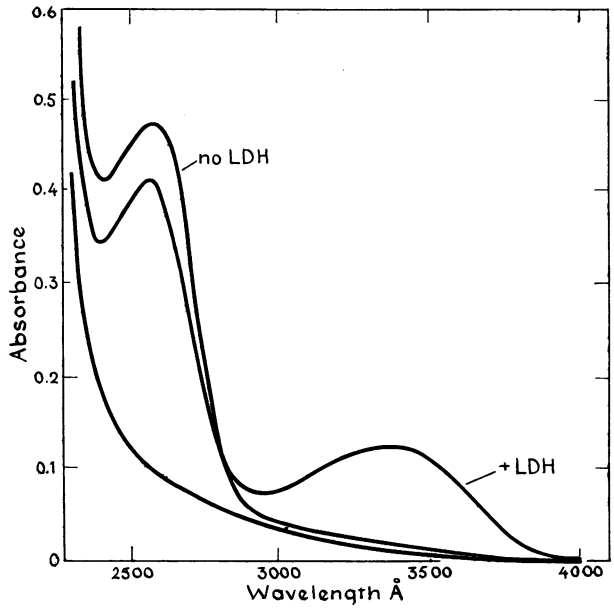

FIG. 1.—Absorption spectrum of purified cofactor before and after treatment with L-lactate and lactic dehydrogenase (LDH). Enzymatic reduction of the Sephadex G-10 fraction was carried out in a reaction mixture (1.0 ml) containing 0.09 *M* glycine-NaOH buffer, pH 10.2, 0.025 *M* sodium lactate, 160 units of muscle lactic dehydrogenase (as measured in the forward reaction[3]) and 0.05 ml of Sephadex G-10 fraction (A_{260} = 8.0). Absorption spectra were measured with a Cary model 14 recording spectrophotometer before addition of enzyme, then after incubation with enzyme for 10 min at room temperature. The base line is the spectrum of a reaction mixture lacking the Sephadex G-10 fraction.

It is likely that most, and perhaps all, of the cofactor activity associated with the crude cell extract is due to DPN. Thus, after treatment of the extract (0.2 ml, A_{260} = 318) with levels of venom phosphodiesterase, potato nucleotide pyrophosphatase or *N. crassa* DPNase which produced complete inactivation of known DPN and the Sephadex G-10 fraction (see above), less than 1 per cent of the initial cofactor activity remained.

TABLE 2

COFACTOR ACTIVITY OF DPN AND DPN ANALOGUES

Compound	Relative activity (per cent)
DPN	100
DPNH	25
Sephadex G-10 fraction	100
Sephadex G-10 fraction + LDH and L-lactate	19
TPN	1
Thionicotinamide-DPN	18
3-pyridine aldehyde-DPN	<1
3-acetyl pyridine deamino-DPN	<1
Deamino-DPN	<1
AppA	<1

Cofactor activity was measured as described under *Materials and Methods*. DPN and its analogues were added at a concentration of 1.5×10^{-7} *M*. Enzymatic reduction of DPN and the Sephadex G-10 fraction with lactic dehydrogenase (LDH) and lactate was carried out as described in the legend to Fig. 1. The values shown are relative to DPN (0.73 $\mu\mu$mole of P^{32}-dT converted to a phosphatase-insensitive form) which was set at 100%.

Cofactor activity of DPN analogues: The cofactor activity of several DPN analogues was examined (Table 2). Of the compounds tested, only DPNH and the thionicotinamide derivative of DPN were active. The lower activity relative to DPN observed with these compounds was due to their significantly higher Michaelis constants. Compared with a K_m for DPN of 1.1×10^{-7} M, the K_m for thionicotinamide-DPN was 6.6×10^{-7} M, and for DPNH, it was 7.0×10^{-7} M. It is noteworthy that the K_m for DPN is of the same order of magnitude as that for the dT substrate (approximately 2.5×10^{-8} M dT chains).

Some activity was observed with relatively high concentrations of TPN (apparent K_m of 5×10^{-5} M); however, this level of activity might be anticipated if the TPN preparation were contaminated with as little as 0.2 per cent DPN. Phosphatase treatment of the TPN (causing its conversion to DPN) resulted in a large increase in cofactor activity, to a level identical with that of DPN. The remaining analogues, in which the adenine moiety or the amide group of the nicotinamide ring were altered, showed no significant activity at concentrations up to 2×10^{-5} M.

The mechanism by which DPN participates in the synthesis of phosphodiester bonds linking dT chains is as yet unknown. Although a DPN-linked oxidation-reduction reaction involving the enzyme itself or some component closely associated with it has not been eliminated, it is quite possible that the energy required to synthesize the new phosphodiester bond may be derived from the pyrophosphate bond of DPN, a bond with a free energy of hydrolysis similar to that of the pyrophosphate bond of ATP.[9] The reaction would then be expected to result in the cleavage of DPN to adenosine 5′-monophosphate and nicotinamide mononucleotide. If so, the polynucleotide-joining enzyme would be unusual in its use of a pyridine nucleotide coenzyme for a reaction not involving oxidation or reduction. The report by Chambon *et al.* is of interest in this regard.[10] They have found that DPN may serve as the immediate precursor of a polymer of adenosine diphosphate ribose in a transglycosylation reaction catalyzed by an enzyme from animal cell nuclei.

Summary.—DPN has been identified as a cofactor for the polynucleotide-joining enzyme from *E. coli*. The apparent K_m (1×10^{-7} M) is of the same order of magnitude as the K_m for the polydeoxythymidylate substrate (2.5×10^{-8} M). DPNH and the thionicotinamide derivative of DPN while active had K_m values sevenfold greater than DPN; other analogues of DPN, including TPN, were inactive.

Note added in proof: Recent studies with P^{32}-DPN indicate that for each equivalent of phosphodiester bond synthesized in the polynucleotide-joining reaction, one equivalent of DPN is converted to adenosine 5′-monophosphate and nicotinamide mononucleotide. Zimmerman *et al.* (these PROCEEDINGS, this issue, p. 1841) have found similar results.

In the absence of polynucleotide, the enzyme catalyzes an exchange reaction between DPN and nicotinamide mononucleotide (but not adenosine 5′-monophosphate), suggesting that an adenylate-enzyme intermediate is formed.

* This work was supported in part by research grants from the National Institutes of Health, USPHS.

† Postdoctoral fellow of the Damon Runyon Memorial Fund for Cancer Research.

[1] Olivera, B. M., and I. R. Lehman, these PROCEEDINGS, **57**, 1426 (1967).

[2] Inactivation of very dilute solutions of enzyme during incubation, noted earlier, can be prevented by including bovine plasma albumin (0.05 mg/ml) in the reaction mixture and diluting the

enzyme in a solution composed of 0.01 *M* Tris-HCl, pH 8.0, 0.002 *M* $MgCl_2$, 0.001 *M* EDTA, and albumin (1.0 mg/ml).

[3] Kornberg, A., in *Mtehods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1955), vol. 1, p. 441.

[4] Anderson, B. M., C. J. Ciotti, and N. O. Kaplan, *J. Biol. Chem.*, **234**, 1219 (1959).

[5] Razzell, W. E., and H. G. Khorana, *J. Biol. Chem.*, **234**, 2105 (1959).

[6] Kaplan, N. O., S. P. Colowick, and A. Nason, *J. Biol. Chem.*, **191**, 473 (1951).

[7] Kornberg, A., and W. E. Pricer, Jr., *J. Biol. Chem.*, **182**, 763 (1950).

[8] One unit of phosphodiesterase activity is that amount which catalyzes the hydrolysis of 1 μmole of p-nitrophenyl 5′-deoxythymidylate in 1 min at 37° (Razzell, W. E., and H. G. Khorana, *J. Biol. Chem.*, **234**, 2105 (1959)).

[9] Kornberg, A., *J. Biol. Chem.*, **182**, 779 (1950).

[10] Chambon, P., J. D. Weill, J. Doly, M. T. Strosser, and P. Mandel, *Biochem. Biophys. Res. Commun.*, **25**, 638 (1966).