BOUND TPN AS THE DETERMINANT OF POLYMORPHISM IN METHEMOGLOBIN REDUCTASE*

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SUMMARY: The pyridine nucleotide-dependent, dye-linked methemoglobin reductase from human erythrocytes can be resolved chromatographically or electrophoretically into two principal forms (I and II). Form (II), which migrates more rapidly than (I) toward the positive electrode during electrophoresis at pH 8.3, contains 1 mole of non-covalently bound TPN per mole of protein (MW = 22,000) as judged by spectral characteristics (absorbance maxima at 327 and 340 nm when the protein is treated with cyanide and hydrosulfite, respectively) and by analysis (thin-layer chromatography and coenzymatic activity with isocitrate dehydrogenase) of the nucleotide released by heat denaturation. TPN can also be displaced by treatment of (II) with *p*-chloromercuriphenylsulfonate. Incubation of (I) with TPN at neutral pH results in the formation of (II).

In a procedure developed recently for purification of the pyridine nucleotide-linked, dye-dependent methemoglobin reducing system from human erythrocytes (1), one of the steps (chromatography on CM-Sephadex) resolved the enzyme into two principal forms. After purification to homogeneity, each form was shown to have essentially the same molecular weight (ca. 22,000) and the same ratio of catalytic activities (ca. 5 and 1.5 μmoles of ferriheme reduced/min/mg protein when DPNH and TPNH were used as reductants). These forms (designated I and II) could also be distinguished by polyacrylamide electrophoresis using the Tris-glycine buffer system (pH 8.3) of Ornstein (2) and Davis (3). Under these conditions, (II) has the faster mobility toward the

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positive electrode. This technique, in addition, revealed the presence of several minor, catalytically active bands ahead of each principal component.

The present investigation was undertaken in order to determine the basis for the chromatographic and electrophoretic differences between (I) and (II). An important lead was obtained from a comparison of the absorption spectra of these forms (cf. Fig. 8 in ref. 1). (I) had the spectrum of a typical protein (λ_{max} at 278 nm) while in (II) the absorbance maximum was shifted to 268 nm, suggesting that the latter form might contain some bound, non-protein component. Although treatment with Dowex-1 failed to alter the electrophoretic mobility of (II), heat denaturation of the protein released a 260 nm-absorbing material into the supernatant. This was subsequently identified as TPN (R_f = 0.13) by thin-layer chromatography (Polygram CEL-300) using ethanol:1 M ammonium acetate, pH 7.5 (55:45) as the solvent system, by changes in its spectrum following treatment with cyanide or hydrosulfite (see below for similar results with the intact protein), and by its coenzymatic activity when assayed by the isocitrate dehydrogenase system (4). From these results, and using a value of 22,000 for the molecular weight of the protein (1), the mole ratio of TPN:protein in (II) varied between 0.7 and 0.8 in different samples. Since this figure would tend to be low due to incomplete release of the nucleotide and/or its destruction in the heat-denaturation step, it is likely that the mole ratio is actually 1.0.

The existence of bound TPN could be further verified by experiments with intact form (II). For example, addition of KCN caused a decrease in the 268 nm absorbance and the appearance of a new band at 327 nm (Fig. 1). Using the extinction coefficient for the cyanide comples of free TPN (ε_{327} = 6.0 x 10^3 M^{-1} cm^{-1} (5)), the data in Fig. 1 can be used to calculate that the TPN:protein ratio in form (II) is 0.9. In a similar experiment (not shown in the Fig.), an absorbance increase at 340 nm was observed upon addition of hydrosulfite to (II) The KCN reaction also provided a convenient method for detecting form (II) on polyacrylamide gels. Treatment of the latter with 0.05 M KCN (prior to staining for protein or enzymatic activity) converted (II) to a fluorescent deriva-

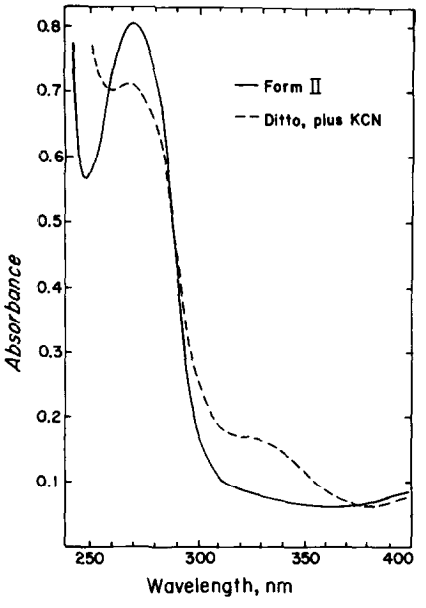

Fig. 1. Absorption spectra of native and cyanide-treated form (II) of methemoglobin reductase. Solid line, spectrum of form (II)(0.45 mg in 0.8 ml of 0.03 M phosphate buffer, pH 7, plus 0.2 ml of H_2O) recorded with a Cary spectrophotometer, Model 14 against a blank containing only buffer; light path, 1 cm. Dashed line, ditto except that 0.2 ml of 5 M KCN was added instead of water.

tive that could be visualized under ultraviolet light. Finally, reaction of (II) with isocitrate and isocitrate dehydrogenase also produced an increase in the 340 nm absorbance, but the rate was about 25-fold slower than that observed with the same amount of free TPN. From the above results, it was concluded that binding of TPN to the protein (although sufficiently strong to survive the purification procedures) was not covalent and that it did not affect the ability of the nucleotide to form a cyanide complex or to be reduced chemically and enzymatically.

Synthesis of an "artificial" (II), identical in all respects to naturally-occurring (II), could be achieved by incubating (I) with TPN at neutral pH. This transformation is illustrated in Fig. 2 (cf. panels A and D). Although not shown in this figure, the mobility of naturally-occurring (II)(cf. Figs. 4, 9 or 10 in ref. 1) is the same as that of synthetic (II) in panel D. Under similar conditions, DPN or DPNH (panels B and C) produced no change in the mobility of (I). However, an interesting result was obtained when (I) was treated with TPNH (panel E). The faster-moving band (II') corresponded, presumably, to an enzyme-TPNH complex (which was fluorescent under ultraviolet light), but this was

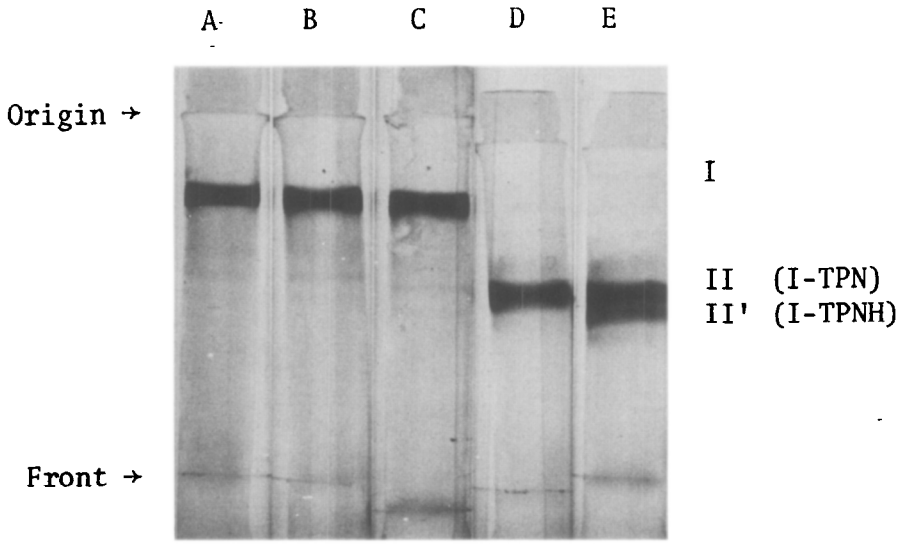

Fig. 2. Conversion of form (I) to form (II) of methemoglobin reductase. Polyacrylamide gel electrophoresis was carried out by methods of Ornstein (2) and Davis (3). Enzyme activity was detected by the staining procedure of Kaplan and Beutler (7), as modified in this laboratory (1). Solutions (0.4 ml) of form (I)(1.0 mg/ml in 0.03 M phosphate buffer, pH 7) were treated with 0.5 mg of the following nucleotides and allowed to stand for 12 hours at 4° before 0.1 ml aliquots were applied to the gels: Panel A, none; B, DPN; C, DPNH; D, TPN; and E, TPNH.

accompanied by an appreciable amount of (II). It is probable that the TPN required for the latter band was generated by the enzyme-catalyzed air-oxidation of TPNH. The ability of the enzyme to bind TPN or TPNH (but not DPN or DPNH) tightly enough to survive electrophoresis is consistent with previously-determined K_m and K_I values for these four pyridine nucleotides (6).

Because the catalytic activities of (II) have been found to be less sensitive than (I) to *p*-chloromercuriphenylsulfonate (*p*CMS)(ref. 7 and unpublished results), the effect of this mercurial upon the (I) → (II) transformation was studied. When (I) was treated with *p*CMS prior to electrophoresis, the resulting complex had a mobility intermediate between that of (I) and (II) (cf. panels A, B and C in Fig. 3). Incubation of the (I)-*p*CMS complex with TPN (panel D) did not lead to any formation of (II). However, when (I) was treated with TPN prior to the *p*CMS (or when II was treated with *p*CMS), a mixture of (II) and the (I)-*p*CMS complex was obtained (panel E). With longer periods of time, the (I)-*p*CMS complex is the sole product. Although not excluding the

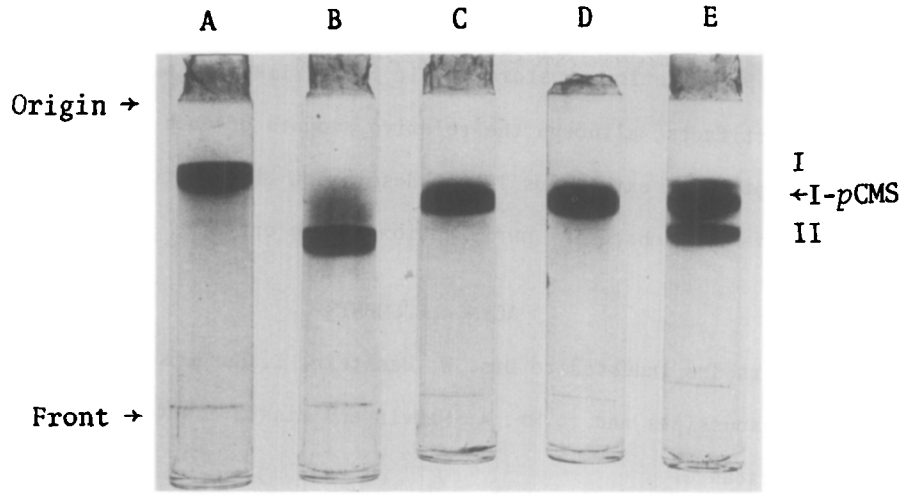

Fig. 3. Effect of *p*CMS upon the conversion of form (I) to form (II) of methemoglobin reductase. Polyacrylamide gel electrophoresis was carried out as described in Fig. 2, except that the protein-staining technique with Amido Black was used. Aliquots (0.09 ml) of form (I)(1.0 mg/ml in 0.03 M phosphate buffer, pH 7) were placed at the top of the acrylamide columns and the following additions were made just before beginning the electrophoresis: Panel A, none; B, 0.02 ml of 1 mM TPN; C, 0.02 ml of 1 mM *p*CMS; D, *p*CMS and, after 5 min, TPN; and E, TPN and, after 5 min, *p*CMS.

possibility that sulfhydryl groups may be involved in the electron transport sequence of the enzyme, these experiments suggest that such groups have a specific function in the binding of TPN or TPNH. They may also serve a similar function in the interaction of DPN and DPNH with the enzyme (although, as shown in Fig. 2, this cannot be demonstrated electrophoretically), since mercurials inhibit both the TPNH- and DPNH-methemoglobin reductase activities (8).

Multiple forms of various methemoglobin reducing systems have been described previously by several laboratories (reviewed in ref. 1). The present experiments establish that insofar as the pyridine nucleotide-linked, dye-dependent enzyme is concerned, the two principal forms of the enzyme (I and II) differ by the absence or presence of an equimolar amount of non-covalently bound TPN. This type of polymorphism is also exhibited by the dihydrofolate reductase from amethopterin-resistant *Lactobacillus casei* (9) except that, in the latter instance, form (II) contains an equimolar amount of TPNH. The nature of the minor sub-bands of methemoglobin reductase is still unexplained, but they may well be artifacts arising from hydrolysis of amide groups on the

protein during purification or storage. It is not likely, however, that (I) and (II) are artifacts, although the relative amounts of each might be altered (e.g. by adsorption of extraneous TPN or desorption of the bound nucleotide) during hemolysis and subsequent purification of the enzyme.

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