SPECTRAL PROPERTIES OF THE COENZYME BOUND TO DOPA DECARBOXYLASE FROM PIG KIDNEY

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1. Introduction

DOPA decarboxylase (EC 4.1.1.26) has been extensively studied, particularly regarding its distribution, specificity and inhibition; unfortunately, not much is known about its mechanism of action, and the mode of binding of the coenzyme (pyridoxal-5'-phosphate) and the substrates. A study of the absorbance properties of the enzyme in the 300–500 nm range, where the bound coenzyme should show its characteristic absorption band, would be highly desirable, since similar studies have provided a good deal of information in other pyridoxal phosphate dependent enzymes [1].

The poor stability of the mammalian enzyme, however, has made the purification and the study of the properties of DOPA decarboxylase a difficult task. Recently, Christenson et al. [2] have obtained a homogeneous preparation from pig kidney; many structural features of the enzyme, but not its absorption spectra, have been described. In a preliminary report, we described a method which gave a highly purified enzyme [3] from the same source.

In this report we shall illustrate the spectral features of the protein-bound coenzyme, showing that at least three different modes of binding may occur.

Coenzyme appears to bind very tightly to the protein; in fact, Christenson et al. [2] were unable to resolve the holoenzyme; in the present paper a method is described to dissociate reversibly the coenzyme from the protein; it will be shown that the reconstitution of the holoenzyme from the apoenzyme and pyridoxal phosphate restores the original spectral features.

Furthermore, the spectral change caused by α-methyl-DOPA provides a clue to explain the peculiar inhibitory action of this substrate analogue [4].

2. Methods

The enzyme was purified from pig kidneys by a method outlined in a previous report [3]; details of the preparation are given elsewhere [5]. The enzyme appeared to be homogeneous in polyacrylamide gel electrophoresis and at least 95% homogeneous in the ultracentrifuge; its coenzyme content (0.94 mole of coenzyme per 112,000 g of protein) is in excellent agreement with the content reported by Christenson et al. (0.7–1.1 mole/112,000 g).

The activity was measured by the method of Christenson et al. (2) but with a shorter reaction time (2 min) and at lower temperature (30°).

Pyridoxamine phosphate was determined by the method of Bossa and Barra [6], after treatment with sodium borohydride [7] to reduce the pyridoxal phosphate present.

3. Results

The absorption spectrum of the purified enzyme in the 300–500 nm region is shown in fig. 1.

Two absorption maxima are present, respectively at 420 and 333 nm. The ratio of the intensity of the two peaks appears to be fairly constant in different enzyme preparations (A_{333}/A_{420} = 3.5–3.8).

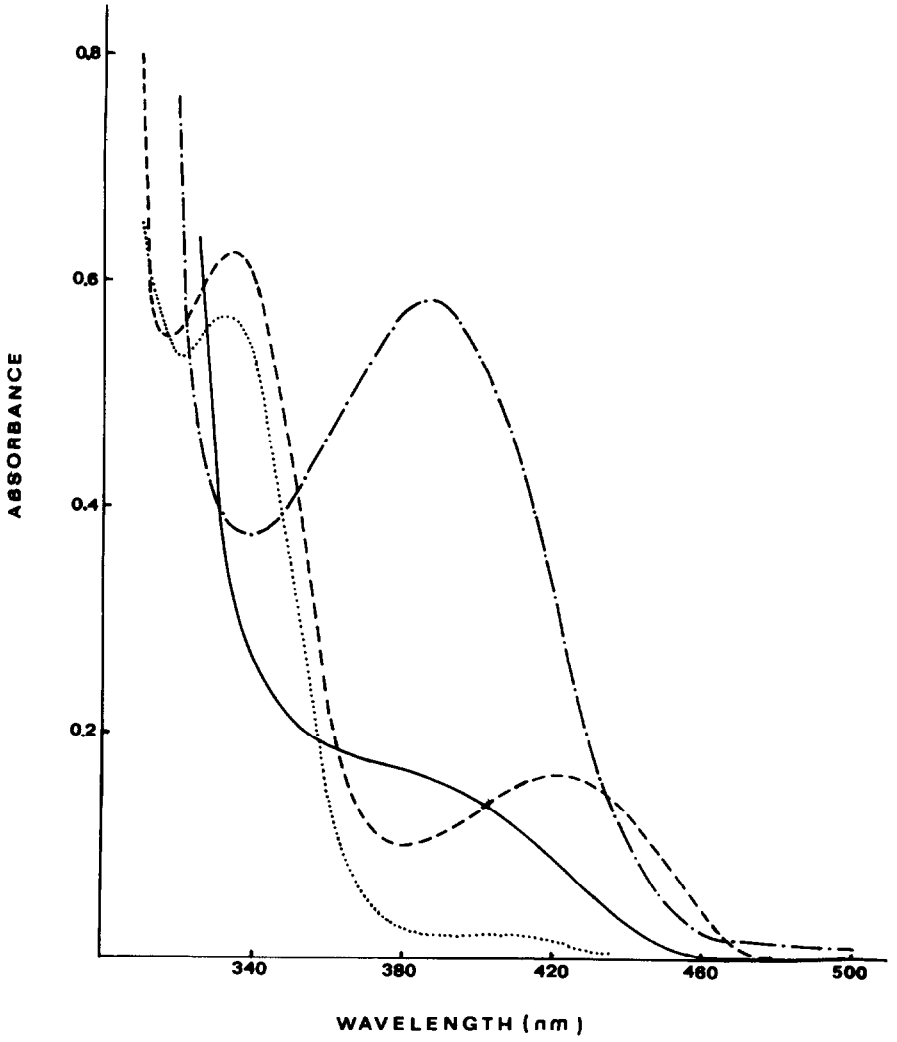

Fig. 1. Absorption spectra of unmodified and reduced DOPA decarboxylase. The enzyme concentration is 10.4 mg/ml. - - - - - -: Enzyme in 0.1 M potassium phosphate buffer, pH 6.8; – : – – – –: enzyme in 0.1 N NaOH; . . . . . .: enzyme treated with 10 mM sodium borohydride; ————— : sodium borohydride-treated enzyme in NaOH 0.1 N.

The absorption spectrum does not change in the pH 7.5–6.0 region, where the enzyme is maximally active towards its best substrate, L-DOPA. After addition of NaOH to a final concentration of 0.1 N the spectrum of free pyridoxal phosphate appears.

The peak at 420 nm, attributable to a Schiff base between the coenzyme and a protein amino group [1] is abolished by the addition of sodium borohydride, which is known to reduce such Schiff bases with the formation of pyridoxyl-compounds; unexpectedly, however, the absorbance at 330 nm, which should increase as a result of the formation of the pyridoxyl compound, is slightly decreased (fig. 1). The addition of NaOH to the reduced enzyme induces the appearance of a small peak at 390 nm, corresponding to about 25% of the original coenzyme; this means that 75% of the total coenzyme has been reduced by sodium borohydride. An interpretation of these data will be given in the discussion.

Addition of hydroxylamine to the enzyme causes

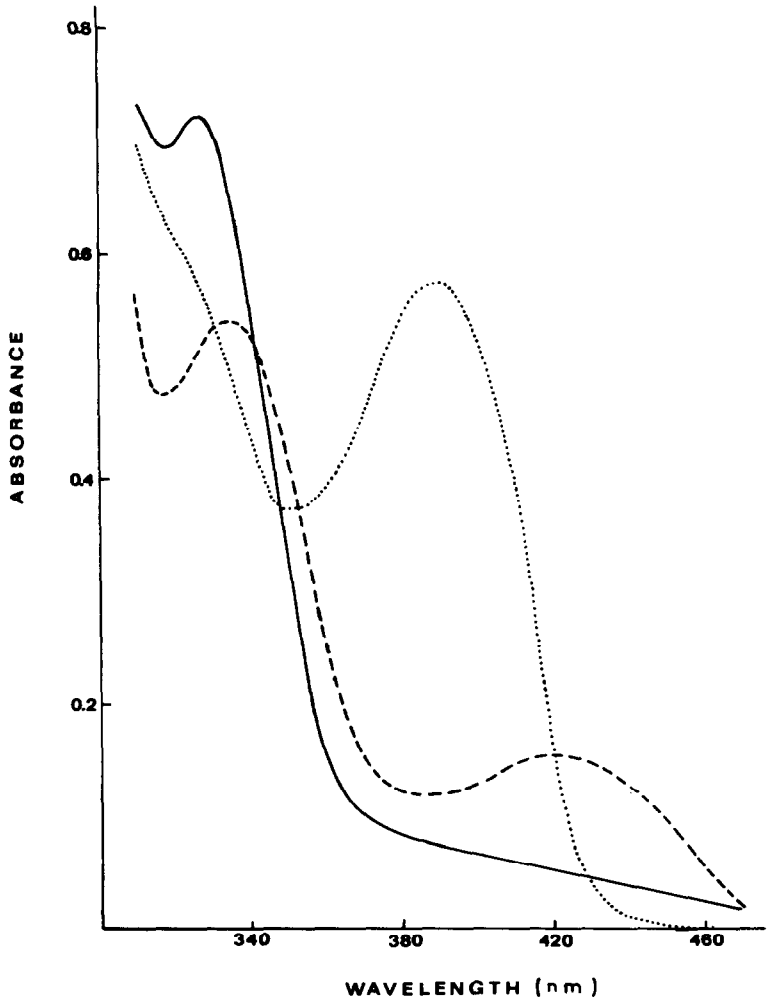

Fig. 2. Effect of hydroxylamine and α-methyl-DOPA on the enzyme. The enzyme (10 mg/ml) was dissolved in 0.1 M potassium phosphate buffer, pH 6.8. - - - - - - : Enzyme; . . . . . : enzyme plus 0.1 M hydroxylamine; ———— : enzyme plus 0.01 M α-methyl-DOPA (3 min after the addition of the inhibitor).

the appearance of a peak at 390 nm (fig. 2) which is quite different from the absorption peak of the oxime formed with free pyridoxal phosphate, which absorbs maximally at 330 nm.

The formation of the oxime with the protein-bound coenzyme is likely to weaken the coenzyme–apoenzyme interaction; resolution of the hydroxylamine-treated holoenzyme has indeed proved possible.

A typical resolution procedure is as follows: the enzyme is dialysed against three changes of a solution 0.1 M of hydroxylamine and 10^{-4} M EDTA, in 0.5 M potassium phosphate buffer, pH 6.5 at 4°; the enzyme is then dialysed against three changes of the same phosphate buffer, containing 10^{-4} M EDTA. Four apoenzyme preparations had residual activities ranging from 0 to 6% of the original value, and after addition of 10^{-6} M coenzyme exhibited activities ranging from 35 to 56% of the original. The failure to obtain the full recovery of the original activity must be attributed to the inherent instability of the enzyme; the apoenzyme should be even more labile, as is the case with other B_6 dependent enzymes.

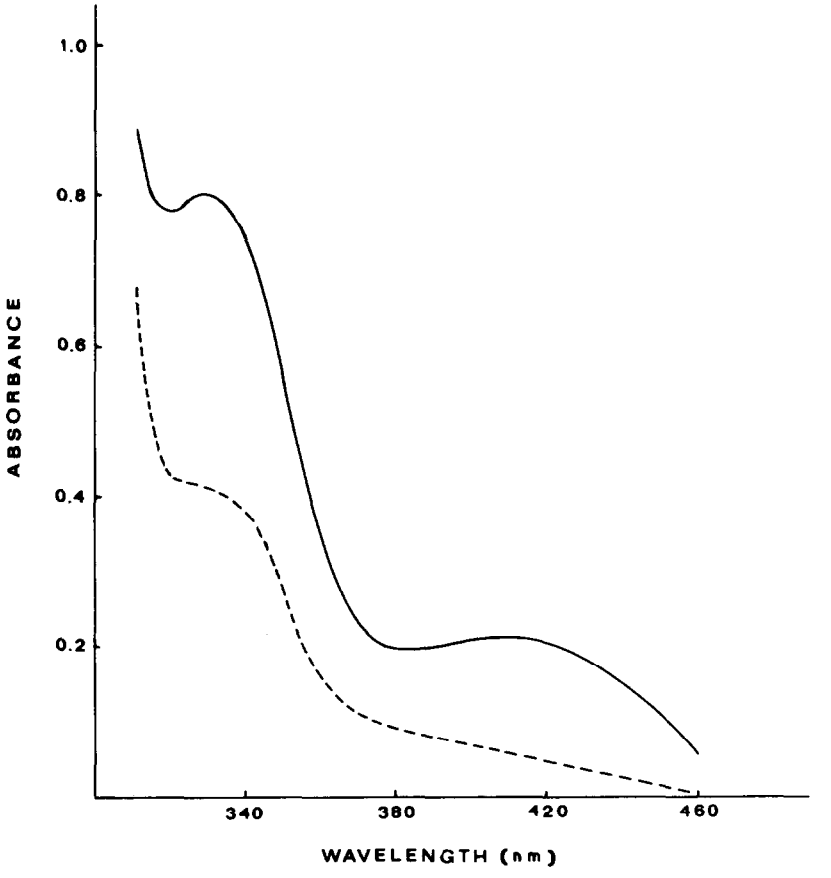

Fig. 3. Spectra of the apoenzyme and reconstituted holoenzyme. The enzyme (28 mg/ml) was dissolved in 0.5 M potassium phosphate buffer, pH 6.5. - - - - - : Apoenzyme; ———— : apoenzyme plus 1.4×10^{-4} M pyridoxal phosphate.

The spectrum of the apoenzyme, shown in fig. 3, has no absorption maxima beyond 300 nm, except for a small shoulder at 330 nm. Upon addition of the coenzyme (half of the enzyme on a molar basis, so as to maintain a slight excess of native apoenzyme) the original spectrum of the holoenzyme is obtained (fig. 3).

The addition of DOPA or Dopamine to the holoenzyme leaves the spectrum practically unchanged. The spectral behaviour is markedly different after the addition of α-methyl-DOPA, which is a powerful inhibitor of DOPA decarboxylation, and undergoes a very slow decarboxylation [2]. α-methyl-DOPA induces a relatively slow ($t_{1/2}$ = 2 min) decrease of the absorption at 420 nm, with a corresponding increase in the 320 nm region (fig. 3). This suggests that at least part of the inhibitory action of α-methyl-DOPA is due to a decarboxylation dependent transamination, analogous to that observed with α-methyl glutamate in the presence of glutamate decarboxylase [8].

To test this hypothesis, an assay for pyridoxamine phosphate has been performed on an α-methyl-DOPA treated enzyme. After 10 min of reaction with 0.01 M α-methyl-DOPA, 0.63 mole of pyridoxamine phosphate were formed per mole of enzyme, proving that transamination had indeed occurred.

4. Discussion

The 420 absorption peak of DOPA decarboxylase, commonly found in most B_6-dependent enzymes [1], is attributable to a protonated Schiff-base in-

volving the aldehydic group of the coenzyme and an amino-group of a protein amino acid residue; in fact it disappears, as expected, following treatment either with $NaBH_4$ or with hydroxylamine.

The peak at 333 nm, also found in many B_6-enzymes [1], is not, instead, unequivocally indicative of a particular coenzyme-protein complex; this absorption band might originate from: (a) a substituted aldimine, usually inactive towards nucleophiles and devoid of coenzymatic activity; (b) a Schiff-base in an apolar medium [9]; (c) other B_6 derivatives lacking a double bond at the 4′ C.

In our case several lines of evidence suggest that this absorption originates from a catalytically active Schiff-base in an apolar medium; (a) the borohydride reduction leaves only 15% of the total coenzyme in a non-reduced state, yet the 333 nm absorbing form, judging from the known absorption coefficient of pyridoxal derivatives, should constitute about 60% of the total coenzyme. This indicates that a large percentage of the species absorbing at 333 nm is affected by reduction; (b) from the spectra of hydroxylamine-treated holoenzyme, and in particular from the intensity of the peak at 390 nm, it can be deduced that hydroxylamine reacts with both the 420 and the 333 nm absorbing forms; (c) resolution of the holoenzyme after hydroxylamine treatment causes a decrease of both the 420 and 333 nm absorbing forms. (d) α-methyl-DOPA transforms over 60% of the coenzyme into the pyridoxamine phosphate form; (e) the fact that borohydride reduction does not increase the 333 nm peak is easily explained if a fraction of the coenzyme is located in an apolar environment, since the produced pyridoxyl compound in such an environment is expected to absorb below 300 nm [9]. Our data do not allow us to establish whether the different modes of binding of the coenzyme originate from the existence of different protein species (e.g. conformers) or rather if an equilibrium is present between the "polar" and "apolar" binding mode. The latter situation has been proposed for tryptophan-synthetase [10], and could have mechanistic implication for the catalysis.

A third, inactive form is present in our enzyme preparation, as shown by a fraction (about 25%) of non-reducible coenzyme, by the small amount of 333 nm absorbing, inactive material present in the apoenzyme and by the incomplete transformation of the coenzyme, caused by α-methyl-DOPA, to pyridoxamine phosphate. This inactive form has properties quite similar to those found in the γ-form of aspartate transaminase from pig heart [11].

Acknowledgements

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