DIRECT SPECTROPHOTOMETRIC ASSAY OF TRYPTOPHANASE

C. H. SUELTER
Department of Biochemistry, Michigan State University, East Lansing, Michigan, 48824, USA

and

Jean WANG and Esmond E. SNELL
Department of Biochemistry, University of California, Berkeley, California, 94720, USA

Received 17 May 1976

1. Introduction

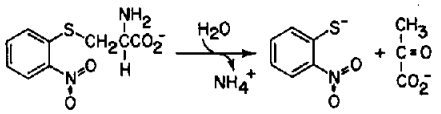
Tryptophanase, a pyridoxal-phosphate dependent enzyme, catalyzes the degradation of tryptophan to yield indole, pyruvate and ammonia. Many other β-substituted amino acids or amino acid derivatives such as cysteine, *S*-alkylcysteines, cysteine sulfinic acid, *S*-benzylcysteine, serine, β-chloroalanine, *O*-benzylserine and α,β-diaminopropionic acid also undergo α,β-elimination to yield pyruvate, ammonia and the β substituent (for a review see [1]). The mechanism of tryptophanase-catalyzed reactions has been studied extensively [1]; the enzyme has also been used for the quantitative estimation of tryptophan and of pyridoxal 5′-phosphate (pyridoxal-P) [1,2], as an aid to classification of bacteria, and more recently for the synthesis of tryptophan or 5-hydroxytryptophan from pyruvate, ammonia and indole or 5-hydroxyindole [3,4]. Tryptophanase is usually assayed by following production of indole from tryptophan in end-point assays [2] or by coupling pyruvate formation to the oxidation of NADH in the presence of excess lactate dehydrogenase (LDH). Since indole strongly inhibits, these assays are linear only for a short time unless indole is removed from solution during assay [2]. The coupled assay also requires additional enzymes and reagents. This paper describes a convenient direct spectrophotometric assay of this enzyme that uses a new, highly reactive chromogenic substrate, *S*-*o*-nitrophenyl-L-cysteine.

2. Materials and methods

S-*o*-Nitrophenyl-L-cysteine and *S*-*p*-nitrophenyl-L-cysteine were prepared by following published methods [5]. All other reagents and chemicals were analytical reagent grade. Tryptophanase was prepared by a modification (Suelter, Wang and Snell, in preparation) of a previous method [3].

3. Results

In the presence of tryptophanase, *S*-*o*-nitrophenyl-L-cysteine (SOPC) undergoes α,β-elimination according to equation 1. SOPC has an absorption maximum in



the visible region at 370 nm, $\epsilon = 2700\ M^{-1}\ cm^{-1}$ (fig.1). The product, *o*-nitrothiophenolate, absorbs maximally at 412 nm (fig.1). The reaction may be monitored at 370 nm with a $\Delta\epsilon = 1860\ M^{-1}\ cm^{-1}$ or at 470 nm with a $\Delta\epsilon = 626\ M^{-1}\ cm^{-1}$. Since the p*K* of *o*-nitrothiophenol is 5.4 (fig.2) the extinction coefficient for the product must be corrected when the assay is carried out below pH 8.0.

Double reciprocal plots [6] of initial velocity as a function of substrate concentration [fig.3] show

 North-Holland Publishing Company – Amsterdam

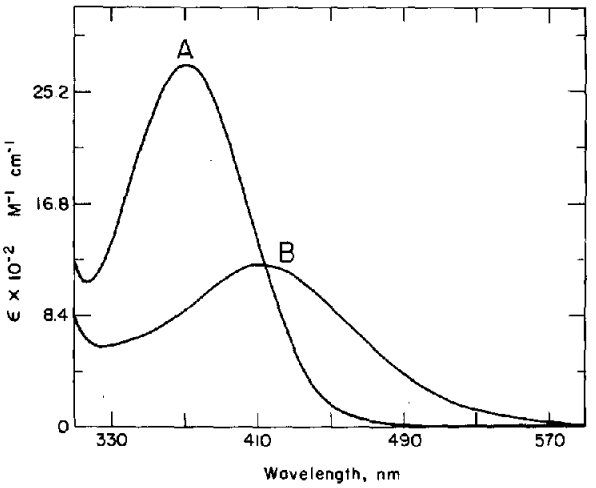


Fig.1. The absorption spectrum of (A) *S*-*o*-nitrophenyl-L-cysteine (SOPC) and (B) *o*-nitrothiophenol in 50 mM triethanolamine·HCl, pH 8.0, 0.1 M KCl, 25°C. Tryptophanase was added to 2.27 × 10^{-4} M SOPC (spectrum A) and the reaction was allowed to proceed to completion. Spectrum B was then recorded.

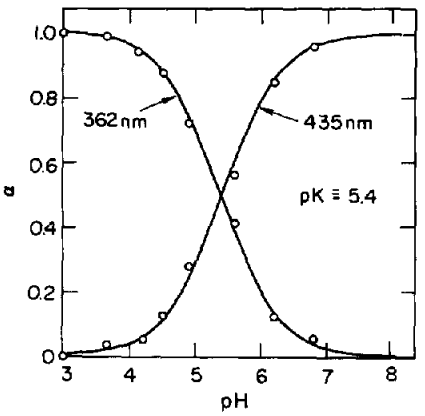


Fig.2. The fractional ionization, α, of *o*-nitrothiophenol as a function of pH. Tryptophanase was added to 2 mM SOPC in 50 mM triethanolamine-0.1 M KCl, pH 8.0. After the reaction had proceeded to completion, samples of the reaction mixture were diluted to give 0.2 mM *o*-nitrothiophenol in 0.1 M buffers of the indicated pH values before the spectra were taken. A single, sharp isosbestic point (at 402 nm) indicated that a single ionizable group was being titrated. Buffers used were potassium formate (pH 3 to 3.65), potassium acetate (pH 4.2–5.6) and potassium 2-(*N*-morpholino)ethane sulfonate (pH 6.2 and 6.8).

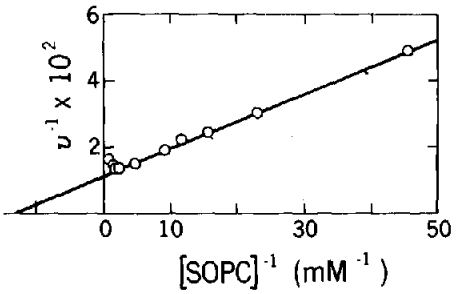


Fig.3. A double-reciprocal plot relating rate of decomposition of SOPC to its concentration. The assay was performed as described in the text in 50 mM potassium phosphate, pH 8.0, and 25°C. Substrate inhibition is observed at concentrations greater than 0.6 mM.

substrate inhibition at [SOPC] > 0.6 mM. As a result, routine assays for tryptophanase are made at 0.6 mM SOPC (~90% saturating). The K_M for SOPC is 0.05–0.06 mM, V_{max} = 60–65 μmol min^{-1} mg^{-1} at 25°C. *S*-*p*-Nitrophenyl-L-cysteine has a K_M of about 3.6 mM and a V_{max} of 40 μmol min^{-1} mg^{-1}. V_{max} for both nitro compounds at 25°C is greater than that for tryptophan (V_{max} = 9 μmol min^{-1} mg^{-1}; K_M = 0.33 mM [1]); however, because of its higher K_M value (3.6 mM) and low solubility, *S*-*p*-nitrophenyl-L-cysteine is not a useful substrate for routine assays. The marked difference between the K_M values of the two nitro compounds can be accounted for by the closer resemblance of SOPC to the native substrate, tryptophan; a nitro group at the ortho position appears to simulate the indole ring of tryptophan (cf. equation 1) better than one at the para position. *S*-2,4-Dinitrophenyl-L-cysteine decomposes spontaneously at pH 8.0 at 25°C, thus negating its use as a substrate.

Free pyridoxal-P and SOPC react in solution with consequent changes in absorbance at both 370 and 470 nm. This is not a problem for normal assays since it is not necessary to add pyridoxal-P to the reaction mixture.

4. Discussion

The assay of tryptophanase using SOPC is direct and spectrophotometric. Since at pH 8.0 the absorp-

tion curves of the substrate, SOPC, and the product of the reaction, *o*-nitrothiophenol overlap, it becomes necessary to follow the decay of substrate at 370 nm or formation of product at 470 nm. Our usual assay, carried out by following decrease of absorption at 370 nm with $\Delta\epsilon = 1860\ M^{-1}\ cm^{-1}$, is not as sensitive as the LDH-coupled tryptophanase assay ($\Delta\epsilon$ of $6200\ M^{-1}\ cm^{-1}$) but is much more convenient and presents fewer complications. A pK = 5.4 for the *o*-nitrothiophenolate ion requires that assays conducted below pH 8 be corrected for the change in ionization.

Because of the wide latitude of substrates acted upon by tryptophanase [1], a great many different assay procedures for this enzyme are possible. Others that have been considered but not developed are: following dechlorination of β-chloroalanine with a Cl^- electrode or a pH stat; following the elimination of pyridinethiol from *S*-pyridyl-L-cysteine by spectrophotometric methods; or coupling the elimination of mercaptans from cysteine or *S*-alkylcysteines to the non-enzymatic reduction of DTNB [7] (D. June and C. H. Suelter, unpublished) or dithiopyridines [8].

Acknowledgements

This work was supported in part by Grant BMS 74 20107 from the National Science Foundation (CHS) and Grant AM 01448 and AF 01575 of the National Institutes of Health (EES). One of us (CHS) acknowledges receipt of a Career Development Award 1 K3 GM 9725 of the National Institutes of Health.

References

[1] Snell, E. E. (1975) Adv. in Enzymol. 42, 287–333.
[2] Haskell, B. E. and Snell, E. E. (1972) Anal. Biochem. 45, 567–576.
[3] Watanabe, T. and Snell, E. E. (1972) Proc. Nat. Acad. Sci. 69, 1086–1090.
[4] Nakazawa, H., Enei, H., Okamura, S., Yoshida, H. and Yamada, H. (1972) FEBS Lett. 25, 43–45.
[5] Boyland, E., Manson, D., Nery, R. (1962) J. Chem. Soc. (London), 606–611.
[6] Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 658–666.
[7] Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70–77.
[8] Grassetti, O. R. and Murray, J. F. Jr. (1967) Arch. Biochem. Biophys. 119, 41–49.