4-FLUOROTRYPTOPHAN ALKALINE PHOSPHATASE FROM E. coli: PREPARATION, PROPERTIES, AND ^{19}F NMR SPECTRUM

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SUMMARY: A procedure is described for the incorporation of 4-fluorotryptophan and 3-fluorotyrosine into E. coli alkaline phosphatase which gives yields comparable to that of the normal enzyme. The spectral and kinetic properties of the fluorinated enzymes are described. The specific activity of the 4-fluorotryptophan enzyme is about 15% higher than for the normal and fluorotyrosine enzymes. The ^{19}F nmr spectrum of 4-fluorotryptophan alkaline phosphatase exhibits three resolved resonances corresponding to the four known tryptophan residues per subunit.

The idea of in vivo incorporation of a fluorine-labeled amino acid into a protein for the purpose of extracting structural information from its ^{19}F nmr spectrum was suggested several years ago (1). There exists, however, a practical difficulty in obtaining a reasonable yield of protein with a high level of incorporation due to the general inhibitory effects on cell growth of most fluorinated amino acid analogs (2). This difficulty can be overcome by choosing an enzyme such as E. coli alkaline phosphatase, whose synthesis can be induced to coincide with the introduction of an analog into the growth medium. Such a technique was used by others to incorporate p-fluorophenylalanine (3) and 3-fluorotyrosine (4) into alkaline phosphatase. The fluorine containing enzymes produced in these studies had properties which differed little, if any, from those of the native enzyme. The purpose of this communication is to describe the biosynthesis,

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properties, and ^{19}F nmr spectrum of 4-fluorotryptophan alkaline phosphatase. The biosynthetic methods developed were also used to prepare the 3-fluorotyrosine enzyme in much higher yield than previously reported (4).

MATERIALS AND METHODS

3-Fluoro-DL-tyrosine was purchased from Cyclo Chemical Company and 4-, 5-, and 6-fluorotryptophan from Sigma Chemical Company. Fluorotyrosine and fluorotryptophan alkaline phosphatase were isolated from E. coli strain H677 (E. Coli Genetic Stock Center No. 4900) (5). This strain is both a tyrosine and tryptophan auxotroph and also has an absolute requirement for histidine, thiamine, and purines. Cells were grown aerobically at 37 °C in a pH 7.8 medium consisting of 0.12 M tris, 0.08 M NaCl, 0.02 M KCl, 0.02 M NH_4Cl, 1.0 mM $MgSO_4$, 4.0×10^{-2} mM $ZnSO_4$, 2.0×10^{-1} mM $CaCl_2$, 0.4% glucose, 6.0 mM KH_2PO_4, 5 μg/ml thiamine, and 20 μg/ml L-histidine, tryptophan, tyrosine, adenine, and guanine. When the culture reached late exponential phase the cells were harvested by centrifugation and resuspended in the same medium lacking both inorganic phosphate and either tyrosine or tryptophan. After addition of the desired fluorinated amino acid (20 μg/ml), incubation was continued for four hours, at which time the cells were again harvested by centrifugation. The enzyme was then released by converting the cells into spheroplasts by lysozyme treatment (6), and the crude extract was purified according to the method of Csopak et al. (7).

Protein concentrations were measured spectrophotometrically at 280 nm using extinction coefficients determined by amino acid analysis of enzyme solutions of known A_{280} measured at pH 7.4. Alkaline phosphatase activity was measured using the procedure and units of Malamy and Horecker (8). The amount of fluorotryptophan incorporation was determined by alkaline hydrolysis followed by amino acid analysis under conditions which separate tryptophan from fluorotryptophans (1). Since tyrosine and fluorotyrosine cannot be separated under the normal conditions of amino acid analysis, a method for differentiating the two was devised. At pH 8.8, there are substantial differences between the ultraviolet absorption spectra of tyrosine (λ_{max} 274 nm) and fluorotyrosine (λ_{max} 289 nm). It was thus possible to determine the ratio of fluorotyrosine to tyrosine by collecting the tyrosine peak as it was eluted from the ion exchange column of the amino acid analyzer and determining the ultraviolet spectrum of the sample at pH 8.8.

^{19}F Nmr spectra were recorded in 5 mm tubes at 94.1 MHz on a Varian XL-100-15 spectrometer equipped with a Nicolet TT-100 Fourier transform package. The spectrometer was internally locked on the deuterium resonance of the solvent (D_2O), and chemical shifts were determined relative to an external capillary of trifluoroacetic acid. The ambient probe temperature was 30±3°C.

RESULTS AND DISCUSSION

When an exponentially growing culture of E. coli H677 was transferred to a medium containing 3-fluorotyrosine or 4-fluoro-

tryptophan instead of the normal amino acid, the pattern of growth switched from exponential to linear and continued for approximately two generations. If the cells were simultaneously deprived of inorganic phosphate, the synthesis of alkaline phosphatase became derepressed, and enzyme containing the fluorinated amino acid was produced (Figure 1). In the presence of 3-fluorotyrosine more enzyme activity was pro-

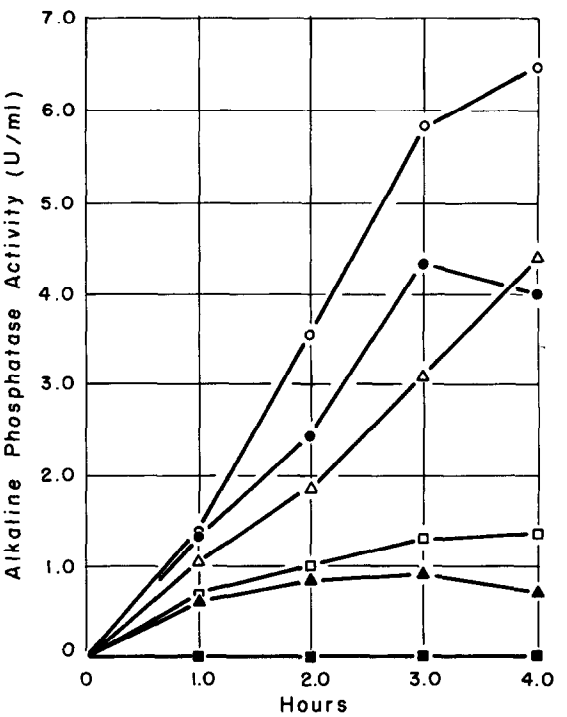

Figure 1. Effect of fluorinated tyrosine and tryptophan analogs on alkaline phosphatase synthesis in an E. coli strain requiring both tyrosine and tryptophan. Exponentially growing cells were harvested, washed, and resuspended in the same volume of medium lacking tyrosine, tryptophan, and inorganic phosphate. Additions of DL-amino acids or amino acid analogs were made to a final concentration of 0.1 mM, and 2 ml aliquotes were periodically withdrawn, sonicated, and assayed for enzyme activity. Alkaline phosphatase activity was determined as a function of time for addition of tryptophan and 3-fluorotyrosine (open dots), tyrosine and tryptophan (solid dots), tyrosine and 4-fluorotryptophan (open triangles), tyrosine and 5-fluorotryptophan (open squares), and tyrosine and 6-fluorotryptophan (solid triangles). No enzyme activity was observed when no addition of amino acids or analogs was made (solid squares).

duced than in the presence of either 4-fluorotryptophan or the normal amino acids. In contrast, Sykes et al., using a different E. coli strain, obtained only about 10% as much fluorotyrosine enzyme as compared to normal enzyme. Confirming a previous report (9), the addition of 5- or 6-fluorotryptophan to the growth medium led to a substantial decrease in the production of alkaline phosphatase activity. 4-Fluorotryptophan, however, proved far superior to the other two isomers, in agreement with previous findings for β galactosidase activity (1, 10).

Large scale preparations of fluorotyrosine and 4-fluorotryptophan enzyme were carried out as described in Materials and Methods. The yield of pure enzyme obtained from a 12-liter batch of cells was about 40 mg. Purity was judged greater than 95% on the basis of acrylamide gel electrophoresis and amino acid analysis. The efficiency of incorporation of both fluorotyrosine and 4-fluorotryptophan was determined to be about 75%.

TABLE I

Properties of Normal and Fluorinated Alkaline Phosphatase

Enzyme	Extinction Coefficient $A^{0.1\%}_{280}$	A_{280}/A_{260}	Specific Activity (units/mg)
Normal	0.77	1.85-1.95	3400 ± 200
3-Fluorotyrosine	0.79	1.55-1.65	3500 ± 200
4-Fluorotryptophan	0.70	1.35-1.45	3900 ± 200

One consequence of the replacement of aromatic amino acids in a protein by fluorinated analogs is a change in its ultraviolet absorbance characteristics. Table I shows the differences in extinction coefficient and A_{280}/A_{260} for the normal and fluorinated enzymes at pH 7.4. At higher pH values approaching the pK_a of the fluorotyrosine hydroxyl group (8.4), the extinction coefficient of fluorotyrosine-substituted alkaline phosphatase becomes considerably greater than 0.79. As expected, the extinction coefficient and A_{280}/A_{260} are lower for fluorotryptophan alkaline phosphatase than for normal enzyme, reflecting the lower λ_{max} of 4-fluorotryptophan (264 nm) compared to tryptophan (280 nm).

The specific activities of the fluorine-substituted enzymes are also shown in Table I. Confirming Sykes' results (4), the activities of the normal and fluorotyrosine enzymes were found to be essentially the same. The higher amount of alkaline phosphatase activity produced with fluorotyrosine in the growth

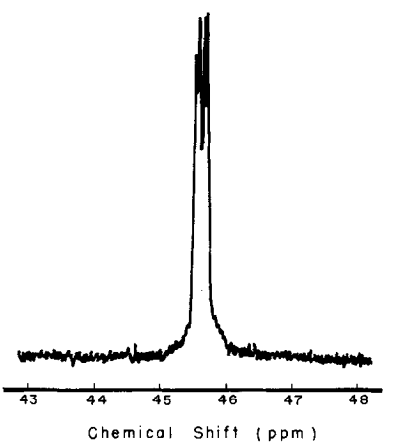

Figure 2. ^{19}F Nmr spectrum of 20 mM 4-fluorotryptophan in 0.3 M tris·HCl (D_2O), pH 7.0; 256 transients, acquisition time 2.0 sec. The observed splitting of the signal results from coupling of the fluorine to hydrogens on the indole ring. The chemical shift scale is in parts per million upfield from the signal of trifluoroacetic acid (external capillary). Note that the chemical shift scale is expanded relative to that of Figure 3.

medium (Figure 1) thus appears to be the result of greater enzyme protein production by this E. coli strain rather than the synthesis of a more active enzyme. The 4-fluorotryptophan enzyme does, however, appear to be more active, consistently giving about 15% higher activity than the normal enzyme.

The ^{19}F nmr spectrum of free 4-fluorotryptophan is shown in Figure 2 and that of 4-fluorotryptophan alkaline phosphatase in Figure 3. In the alkaline phosphatase spectrum, resonances from three classes of tryptophan residues are clearly resolved: a broad peak upfield from the position of the free amino acid and a pair of downfield resonances which together integrate to three times the area of the upfield peak. It is known that there are

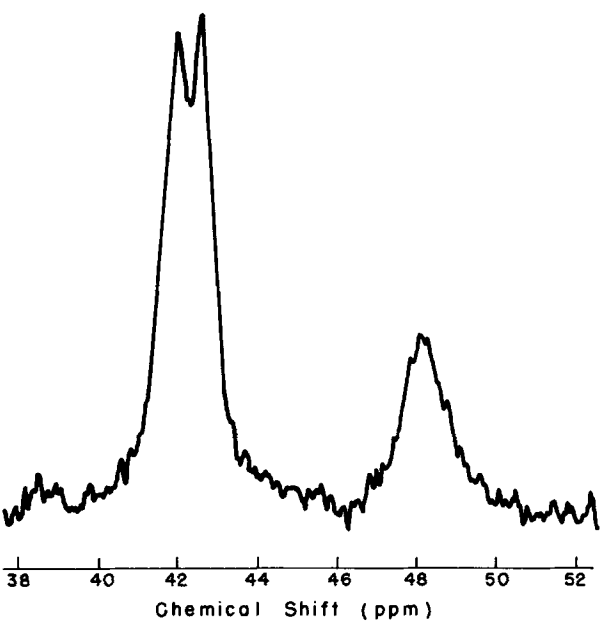

Figure 3. ^{19}F Nmr spectrum of 75 mg/ml 4-fluorotryptophan alkaline phosphatase in 0.2 M tris·HCl (D_2O), 0.2 M NaCl, pH 7.8; 29,292 transients, acquisition time 0.26 sec. A negative exponential corresponding to a line broadening of 3.0 Hz was applied. The lack of observed splitting due to coupling with hydrogens (cf. Figure 2) is attributable in part to the exponential filtering employed and also to a broadening of individual signals which reflects immobilization of fluorine nuclei in the 4-fluorotryptophan enzyme relative to fluorine nuclei in free 4-fluorotryptophan.

four tryptophan residues per subunit (R. A. Bradshaw, private communication). The environments of tryptophan residues are thus sufficiently different in the native folded structure that three of the four can be easily resolved by ^{19}F nmr. The spectrum of the fluorotyrosine enzyme (not shown) was identical to that reported by Sykes et al. (4, 11).

The great sensitivity of ^{19}F chemical shifts to environment in proteins has been demonstrated previously for fluorotyrosine-labeled alkaline phosphatase (4,11) and is illustrated here again for the fluorotryptophan-labeled enzyme. Using the techniques described above, these fluorinated enzymes can be easily isolated in the quantities necessary to carry out nmr studies. The ^{19}F probes should prove especially useful for resolving the current controversies about Zn^{2+} and Mg^{2+} function and stoichiometry as well as the existence of subunit interactions in the native dimer. These and other questions are currently under investigation.

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REFERENCES

1. Browne, D.T., Kenyon, G.L., and Hegeman, G.D. (1970) Biochem. Biophys. Res. Commun., 39, 13-19.
2. Marquis, R.E. in Smith, F.A. Ed. (1970) Handbook of Experimental Pharacology, New Series, Vol. XX/2, pp. 165-192, Springer-Verlag, Berlin.
3. Richmond, M.H. (1963) J. Mol. Biol. 6, 284-294.
4. Sykes, B.D., Weingarten, H.I., and Schlesinger, M.J. (1974) Proc. Nat. Acad. Sci. U.S., 71, 469-473.
5. Stouthamer, A.H., de Haan, P.G., and Nijkamp, H.J.J. (1965) Genet. Res., 6, 442-453.
6. Schlesinger, S. (1968) J. Biol. Chem., 243, 3877-3883.
7. Csopak, H., Garellick, G., and Hallberg, B. (1972) Acta. Chem. Scand., 26, 2401-2411.
8. Malamy, M.H., and Horecker, L.B. (1964) Biochemistry, 3, 1893-1897.
9. Munier, R.L., Drappier, A., and Thommegay, C. (1967) C.R. Acad. Sci. (Paris), D265, 1429-1432.
10. Pratt, E.A. and Ho, C. (1975) Biochemistry, 14, 3035-3040.
11. Hull, W.E. and Sykes, B.D., (1974) Biochemistry, 13, 3431-3437.