ACETYLATION OF THE TYROSINE RESIDUES OF HORSE HEART CYTOCHROME c [1]

John R. Cronin[2] and Henry A. Harbury

Department of Biochemistry, Yale University,
New Haven, Connecticut

Received July 12, 1965

In studies of the structure and function of cytochrome c, comparatively limited attention has been given thus far to the role of the aromatic amino acid residues. This role may well be one of considerable consequence, perhaps especially so in relation to energy transfer, and it seems of interest to attempt its further exploration. We report here some initial results of experiments on O-acetylation of the tyrosine residues.[3] The data indicate that: 1.) reaction of horse heart ferricytochrome c with N-acetylimidazole results in acetylation not only of tyrosine, but also of lysine residues, in contrast to findings for carboxypeptidase A (Simpson et al., 1963); 2.) reaction of N-acetylimidazole with the fully guanidinated derivative of horse heart ferricytochrome c (guan-ferricytochrome c),[4] in which this lack of specificity is avoided, leads readily, under the conditions used, to the formation of about two moles of phenyl ester per mole of protein, and four moles per mole of protein when 8 M urea is present; 3.) (ac·tyr)$_2$guan-cytochrome c and (ac·tyr)$_4$guan-cytochrome c display depressed electron transfer

---

1 Supported by grants from the National Institutes of Health (GM-07317) and the National Science Foundation (NB 1556).

2 Postdoctoral Fellow of the National Institutes of Health, U.S. Public Health Service.

3 For results of iodination, cf. Ishikura et al. (1959); for titration studies, cf. Stellwagen (1964), Rupley (1964), and Flatmark (1964).

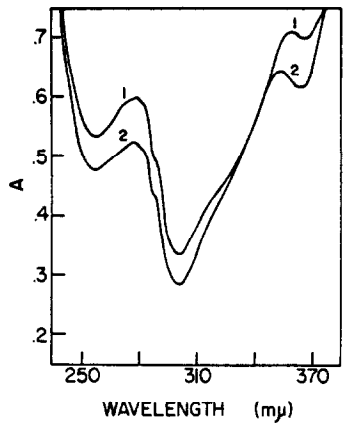
4 Abbreviations: cytochrome c, horse heart cytochrome c; guan-cytochrome c, horse heart cytochrome c with all lysine residues guanidinated; (ac·tyr)$_n$guan-cytochrome c, guan-cytochrome c with n moles of O-acetyltyrosine per mole of protein.

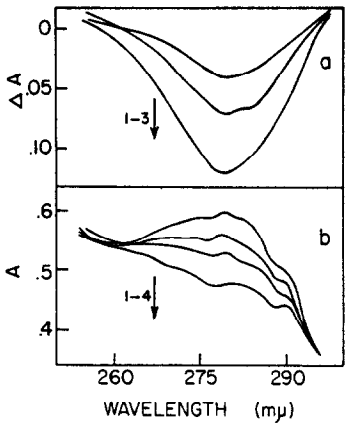
activities in the succinate oxidase system; 4.) extrinsic imidazole coordinates to (ac·tyr)$_2$guan-ferricytochrome c at concentrations much lower than those required in the case of the non-acylated protein, and at these concentrations induces rapid loss of one O-acetyl group; 5.) this conversion of (ac·tyr)$_2$guan-ferricytochrome c to (ac·tyr)$_1$guan-ferricytochrome c is accompanied by a marked increase in electron transfer activity; 6.) brief alkaline hydrolysis restores the electron transfer activity exhibited by the protein in non-acetylated form.

Materials and Methods. Horse heart cytochrome c was obtained from Sigma Chemical Co. (Type III). Guan-cytochrome c (Hettinger and Harbury, 1964) contained less than 0.2 residue of unmodified lysine per mole of protein. N-Acetylimidazole (K and K Laboratories, Inc.) was recrystallized from isopropenyl acetate. N,O-Diacetyltyrosine was obtained from Mann Research Laboratories, Inc. Reaction with N-acetylimidazole (Simpson et al., 1963) was carried out at room temperature in 0.02 M potassium phosphate buffer, pH 7.5, with the protein at a concentration of 3×10^{-4} M, and the initial reagent concentration either 0.02 M or 0.03 M. In a few comparative measurements, 0.02 M sodium Veronal-2 M NaCl, pH 7.5, was used as buffer, and in some experiments 8 M urea was present. After reaction, samples were passed through a column of Sephadex G-25. The number of O-acetyltyrosine residues was determined from the change in absorbance at 278 mμ (Simpson et al., 1963), and from acetohydroxamate formation upon reaction with hydroxylamine at pH 6.5 (Balls and Wood, 1956). Acetylation of threonine (there is no serine or cysteine in the protein) was looked for by reaction with hydroxylamine at pH 11.5 (Balls and Wood, 1956), and acylation of amino groups was followed by reaction with ninhydrin (Moore and Stein, 1954) and by comparison of electrophoretic mobilities. Electron transfer activity was determined as specified previously (Hettinger and Harbury, 1964). Absorption spectra were recorded with a Bausch and Lomb Spectronic 505 spectrophotometer (1-cm cuvettes).

Results. Complex formation with extrinsic imidazole alters the ultra-

violet spectrum of both guanidinated and non-guanidinated ferricytochrome c (Fig. 1).[5] Upon reaction of guan-ferricytochrome c with acetylimidazole, this modification in spectrum is superposed on the change reflecting O-acetylation, but the two effects can be readily resolved by removal of the imidazole with Sephadex G-25 (Fig. 2).





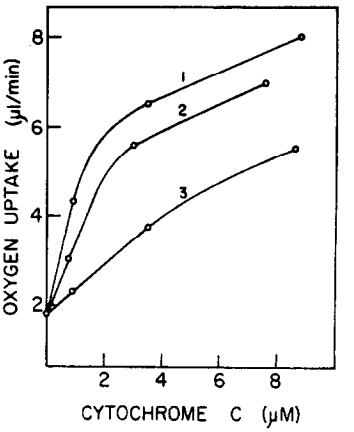


Fig. 1 (left). Ultraviolet spectra of ferricytochrome c (2.5×10^{-5} M) in the absence (1) and presence (2) of 0.29 M imidazole. Phosphate buffer, 0.02 M, pH 7.5.

Fig. 2 (center). (a) Difference spectra of $(ac \cdot tyr)_n$guan-ferricytochrome c versus guan-ferricytochrome c (2.5×10^{-5} M). Phosphate buffer, 0.02 M, pH 7.5. (1), n = 1.1; (2), n = 2.0; (3), n = 3.7. (b) Spectra of $(ac \cdot tyr)_n$-guan-ferricytochrome c (2.5×10^{-5} M). Phosphate buffer, 0.02 M, pH 7.5. (1), n = 0; (2), n = 1.1; (3), n = 2.0; (4), n = 3.7.

Fig. 3 (right). Electron transfer activity of $(ac \cdot tyr)_n$guan-cytochrome c in succinate oxidase system (Schneider and Potter, 1943). (1), n = 0; (2), n = 1.1; (3), n = 2.0.

In representative experiments, the number of acetylated tyrosine residues, as determined from the spectra (s), and the number of acetyl groups reactive towards hydroxylamine at pH 6.5 (h) and 11.5 (h') were found to be (moles per mole of guan-cytochrome c): 1.) for the product of reaction with acetylimidazole for 20 min, followed by passage through Sephadex, 2.1 (s), 2.0 (h), 1.9 (h'); 2.) for the same product, exposed subsequently for 120

---

5 A corresponding change has been observed previously at the weak 695 mμ band of ferricytochrome c (Schejter and George, 1964).

min to 3 x 10^{-3} M imidazole, followed by second passage through Sephadex, 1.1 (s), 1.2 (h), 1.1 (h'); 3.) for the product of reaction with acetylimidazole for 70 min in the presence of 8 M urea, followed by passage through Sephadex, 3.8 (s), 2.8 (h), 3.6 (h').

The conversion of (ac·tyr)$_2$guan-ferricytochrome c to (ac·tyr)$_1$guan-ferricytochrome c attendant upon addition of 3 x 10^{-3} M imidazole proceeded with a half life of approximately 15 min (0.02 M potassium phosphate buffer, pH 7.5, room temperature). This compares with a half life of about 15 hr obtained spectrophotometrically for the conversion, under corresponding conditions, of N,O-diacetyltyrosine to N-acetyltyrosine, and a value in excess of 30 hr, based on acetohydroxamate formation, for loss of the O-acetyl group of (ac·tyr)$_1$guan-ferricytochrome c. Upon the omission of extrinsic imidazole, the rate of deacylation of (ac·tyr)$_2$guan-ferricytochrome c was greatly diminished.

As shown in Fig. 3, (ac·tyr)$_2$guan-cytochrome c, over the range of concentrations used, stimulated oxygen uptake in the succinate oxidase system significantly less than did (ac·tyr)$_1$guan-cytochrome c. This depressed activity did not reflect the presence of the diacetyl derivative in aggregated form (Margoliash and Lustgarten, 1962): its movement on Sephadex G-75 was identical to that of the non-acetylated protein. Electron transfer activity could be restored to that displayed before acylation, with concomitant reversal of the changes in spectrum, by maintenance of the preparation for 10 min at pH 12.

The sample with 3.6-3.8 O-acetyltyrosine residues, obtained upon reaction in the presence of urea, gave rise to essentially the same oxygen uptake as did (ac·tyr)$_2$guan-cytochrome c.

There was no indication that reaction of guan-ferricytochrome c with acetylimidazole led to acetylation of aliphatic hydroxyl groups. The acetohydroxamate values for the product obtained upon reaction in the presence of urea probably are to be accounted for in terms of one of the O-acetyl-

tyrosine residues becoming inaccessible at pH 6.5 in the course of the removal of urea prior to analysis.

Reaction of non-guanidinated cytochrome c with acetylimidazole led to extensive acylation of amino groups. At a 60-fold molar excess of acetylimidazole in phosphate buffer, the ninhydrin color given by the protein was reduced by half, and at a 20-fold molar excess in Veronal-NaCl buffer, a reduction of about 20% was obtained. Comparisons of electrophoretic mobility yielded results consistent with those of colorimetric analysis.

Discussion. The observation that addition of imidazole to $(ac \cdot tyr)_2$guanferricytochrome c results in the loss of one O-acetyl group at a rate much greater than that of the second, and much greater than the rate of hydrolysis of N,O-diacetyltyrosine, suggests that there is in this preparation an O-acetyltyrosine residue in an environment particularly conducive to catalysis of its cleavage. The extrinsic imidazole could, in principle, participate in this catalysis both directly and indirectly, in the latter instance through the induction of a change in structure calling into play a group not operative in this respect in the protein free of added ligand. Such a group could be the imidazole side chain (cf. Bruice and Sturtevant, 1959) displaced from its coordination position by the extrinsic imidazole, or could be, among others, a carboxylate group (cf. Bruice and Pandit, 1960). It would of course be of special interest in relation to the question of possible proximity of tyrosine to the prosthetic group, were the rapid deacylation to reflect a role of a group part of or near the central coordination complex.

The present data do not establish whether the coordination of extrinsic imidazole affects the accessibility of one or more tyrosine residues in the acylation reaction with acetylimidazole, but this could well be the case. It is clear, similarly, that the acylation and deacylation reactions could both be influenced significantly by the change in structure which attends reduction of the molecule. Comparisons of the reactivity of the tyrosine residues in the two states of oxidation of the protein, at different levels of O-acetyla-

tion, may provide useful information.

References

Balls, A.K., and Wood, H.N. (1956), J. Biol. Chem. 219, 245.
Bruice, T.C., and Sturtevant, J.M. (1959), J. Am. Chem. Soc. 81, 2860.
Bruice, T.C., and Pandit, U.K. (1960), J. Am. Chem. Soc. 82, 5858.
Flatmark, T. (1964), Acta Chem. Scand. 18, 1796.
Hettinger, T.P., and Harbury, H.A. (1964), Proc. Natl. Acad. Sci. U.S. 52, 1469.
Ishikura, H., Takahashi, K., and Titani, K. (1959), J. Biochem. 46, 719.
Margoliash, E., and Lustgarten, J. (1962), J. Biol. Chem. 237, 3397.
Moore, S., and Stein, W.H. (1954), J. Biol. Chem. 211, 907.
Rupley, J.A. (1964), Biochemistry 3, 1648.
Schejter, A., and George, P. (1964), Biochemistry 3, 1045.
Schneider, W.C., and Potter, V.R. (1943), J. Biol. Chem. 149, 217.
Simpson, R.T., Riordan, J.F., and Vallee, B.L. (1963), Biochemistry 2, 616.
Stellwagen, E. (1964), Biochemistry 3, 919.