A REVERSIBLE REACTION BETWEEN THE ε - AMINO GROUPS OF CYTOCHROME C AND SALICYLALDEHYDE

J. N. Williams, Jr., and R. M. Jacobs

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland

Received February 14, 1966

The ε - amino groups of proteins can be reacted with various reagents to modify the structure of the protein. With the exception of the trifluoroacetyl derivatives (Goldberger and Anfinsen, 1962; Fanger and Harbury, 1965), the added groups are difficult to remove without disrupting peptide linkages. Even with the trifluoroacetyl derivatives a high pH and lengthy hydrolysis are required to break the trifluoroacetamide linkages, conditions which could result in conformational changes.

It was felt that reversible modification of the ε - amino groups of cytochrome c under very mild conditions might prove to be a useful technique for studies of the structure of this and other proteins. Such a reaction appeared to be possible by Schiff's base (azomethine linkage) formation between ε - amino groups and an added aldehyde. Salicylaldehyde was chosen for a first study since reactions beyond the initial Schiff's base formation would be less likely to occur than with highly reactive aldehydes such as the short chained aliphatics.

It had been expected that if salicylalation of cytochrome c occurred, the product probably would be enzymatically inactive since positive charges on the protein would be lost. Loss of positive charges whether by acetylation (Minakami et al. 1958), trifluoroacetylation (Fanger and Harbury, 1965), and

succinylation or maleylation (unpublished results from this laboratory) produce a loss in enzymatic activity of cytochrome c whereas complete guanidination allows retention of activity (Hettinger and Harbury, 1965).

In the present studies it was found that a maximum of 19 moles of salicylaldehyde are bound per mole of cytochrome c. The product was inactive in the $NADH_2$ - cytochrome c reductase (NCR) system of rat heart. The aldehyde is easily removed by dialysis resulting in a cytochrome c that is still enzymatically inactive but which can be converted completely to the active form by momentary treatment with mild acid or base.

METHODS AND RESULTS

Conditions found to produce the maximal loss of cytochrome c activity in the NCR system were as follows (final concentration of components): 0.00085 M cytochrome c (horse heart, Sigma Type III, assay 98 - 100% pure); 0.33 M K_2HPO_4 (pH 9.6); 0.017 M, or greater, salicylaldehyde adjusted to pH 9.6 with KOH. The mixture was stirred at room temperature for 1 hour. A precipitate of salicylaldehyde - cytochrome c complex forms under these conditions (ionic strength=1). At low ionic strengths a precipitate does not form. The NCR assay was carried out as follows: In a 1 - ml cuvette were mixed 55 μmoles of sodium phosphate (pH 7.4), 1 μmole of $NADH_2$ (pH 7.4), 5 μmoles of KCN, and water to make 0.9 ml. In another cuvette were mixed 25 μmoles of sodium phosphate (pH 7.4), 5 μmoles of KCN, 0.3 ml of a 1% rat heart homogenate in 0.1 M sodium phosphate (pH 7.4), and water to make 0.9 ml. At zero time, 0.1 ml. of the cytochrome c preparation was added to both cuvettes. The cuvettes were mixed by inversion and the absorbance of the second cuvette read against the first at 550 mμ in a recording spectrophotometer for 2 min, to obtain rate of reduction, or after 10 min of standing at room temperature, to obtain the extent of reduction. In most cases the extent of reduction only was measured since there was good correlation between rate and extent of reduction under these conditions. The direct addition of salicylaldehyde to the

NCR system with native cytochrome c added separately had no effect on the activity under these conditions in the range of salicylaldehyde concentration employed in the present study. Bound salicylaldehyde was measured by reacting the sample (plus water to make 2 ml) with 0.5 ml 0.04% 2,4-dinitrophenylhydrazine in 2 N HCl at 70° for 30 min, followed by addition of 1 ml of 2 N KOH. A blank and standard (0.125μmole of salicylaldehyde in 15 λ ethanol) were treated similarly. After 3 min the color was read at 560 mμ against the blank. Total cytochrome was estimated as the pyridine hemochromogen in 1:1 pyridine - 0.2 N KOH plus sodium hydrosulfite.

Fig. 1 shows the effect of increasing salicylaldehyde concentration on

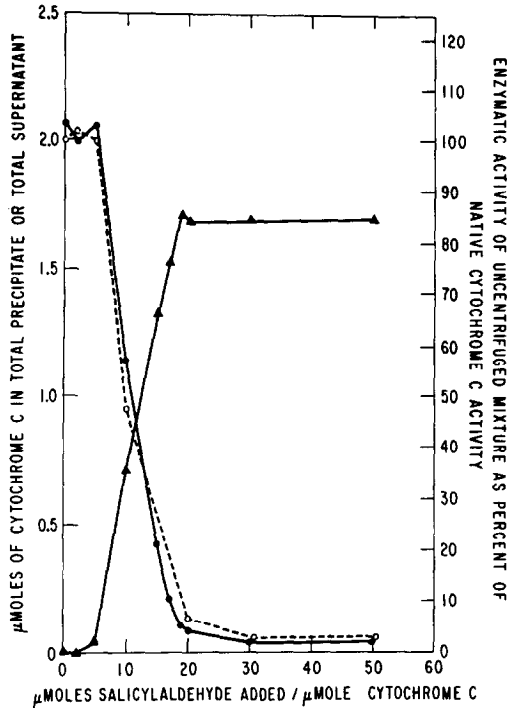

Figure 1 Uptake of salicylaldehyde by cytochrome c and activity of the salicylalated cytochrome c in a heart $NADH_2$-cytochrome c reductase system as a function of μmoles of salicylaldehyde allowed to react with cytochrome c.

▲ = μmoles of cytochrome c in the total precipitate from the reaction mixture.
● = μmoles of cytochrome c in the total supernatant fraction from the reaction mixture.
-○-= activity of an aliquot of the total reaction mixture as per cent of the activity of the same amount of native cytochrome c.

uptake of aldehyde by cytochrome c. After incubating for 1 hr. as described above, the mixtures were centrifuged at 3,000 rpm for 45 min. and the supernatant fraction decanted completely from the precipitate. Here it can be seen that the maximum precipitation of cytochrome c occurred when 19 moles of salicylaldehyde were taken up per mole of protein. Direct analysis of the precipitate also indicated that 18 - 20 moles of aldehyde were bound per mole of protein when 19 or more equivalents of salicylaldehyde were added. Horse heart cytochrome c is known to contain 19 lysine residues (Margoliash et al, 1961). Analysis of each of the mixtures before centrifugation in the NCR assay system gave the results indicated by the broken lines in Fig. 1.

Dialysis of the precipitate (19 moles of aldehyde/mole protein) against

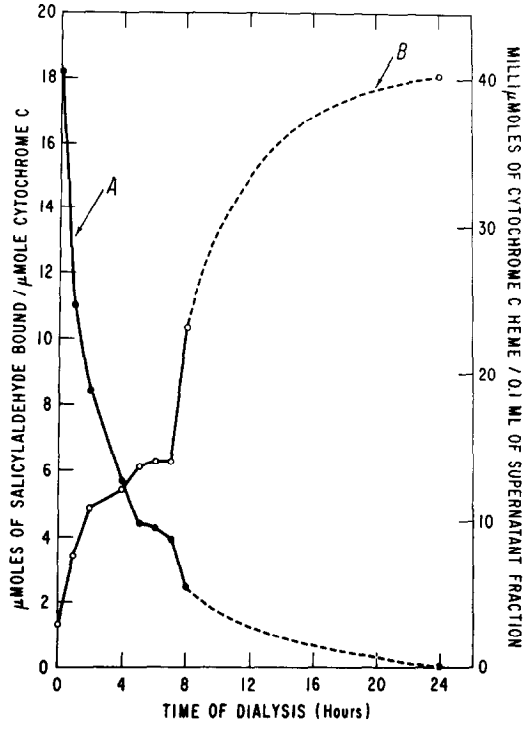

Figure 2 Effect of dialysis of 19:1 salicylaldehyde-cytochrome c precipitate in 0.05 M K_2HPO_4 on (A) μmoles of aldehyde bound /μmole of cytochrome c and (B) solubility of the modified cytochrome c as the dialysis proceeds. Aliquots of the original reaction mixture were dialyzed against 1000 volumes of buffer. Buffer was changed every hour for 8 hours. The points for curve B were obtained by centrifuging aliquots from each dialysis bag and measuring cytochrome c as the pyridine hemochromogen in aliquots from the supernatant fractions.

0.05 M K_2HPO_4 (Fig. 2) slowly removed the salicylaldehyde, with an inflection point from 4-7 hours, when 4 moles of aldehyde were still bound per mole of protein. The insoluble protein gradually became soluble during 24 hours of dialysis as indicated in the figure. None of these dialyzed preparations, however, gave activity in the NCR assay system although ability of the cytochrome to be reduced by ascorbate increased linearly reaching the theoretical value after 24 hours of dialysis. Preliminary studies involving separation of the 24-hour dialyzed, enzymatically inactive sample on Sephadex G 100 suggests the presence of polymers or molecular aggregates which may be similar to those found by Margoliash and Lustgarten (1962). When the 24-hour dialyzed sample was briefly adjusted to pH 3 or below or to pH 11.5 or above, full enzymatic activity was restored. Whether polymers are indeed formed and, if so, what is the mechanism of their formation are questions under investigation at present.

REFERENCES

Fanger, M.W. and Harbury, H.A., Biochemistry, 4 (1965) 2541.
Goldberger, R.F. and Anfinsen, C.B., Biochemistry, 1 (1962) 401.
Hettinger, T.P. and Harbury, H.A., Biochemistry, 4 (1965) 2585.
Margoliash, E. and Lustgarten, J., J. Biol. Chem. 237 (1962) 3397.
Margoliash, E., Smith, E.L., Kreil, G. and Tuppy, H., Nature, 192 (1961) 1125.
Minakami, S., Titani, K. and Ishikura, H., J. Biochem. (Tokyo), 45 (1958) 341.