THE REDUCTION OF CYTOCHROME c BY GLYCINE

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Summary: The rate of the reduction of ferricytochrome c at alkaline pH depends on the composition of the buffer. In glycine-NaOH buffer the reduction is accompanied by the formation of one mole of glyoxylic acid per two moles of cytochrome c reduced. The rates of reduction in glycyl-glycine, triglycine and DL-alanine-NaOH buffers are considerably slower, and in carbonate and Tris are negligible.

The tendency of cytochrome c to undergo spontaneous reduction in weakly alkaline solutions was noticed by Boeri (1) and the reaction was investigated recently by Brady and Flatmark (2). In their studies, performed in glycine-NaOH buffer, these authors followed the kinetics and equilibrium of the "autoreduction" and concluded that "constituents of the polypeptide chain participate in the generation of the reducing equivalents" (2). In this report it will be shown that the rate and extent of reduction of ferricytochrome c at alkaline pH depends on the nature of the buffer. Autoreduction in glycine-NaOH buffer is actually due to reduction of ferricytochrome c by the amino group of glycine, with a concomitant formation of glyoxylic acid.

MATERIALS AND METHODS

Sigma Type III cytochrome c was purified on Amberlite IRC-50 (3). Reduction of the enzyme in different buffers was measured on a Cary 16 spectrophotometer at 550 nm, 37°C.

For the simultaneous determination of the rate of reduction and glyoxylic acid formation, 0.7 mM ferricytochrome c in 0.25 M glycine-NaOH was incubated at 37°C. 0.1 ml aliquots were withdrawn, diluted with 0.9 ml

0.2 M phosphate buffer pH 7.0 and the absorbancies at 550 nm were measured. Calculations were based on a value of 21.3 mM^{-1} for the extinction coefficient of the reduced minus the oxidized form (4). At the same time 0.5 ml aliquots were deproteinized with 0.2 ml 30% trichloroacetic acid. The glyoxylic acid content of the supernatants (0.25 ml) was estimated with two procedures: a) pyrogallol in conc. H_2SO_4 (5) and b) phenylhydrazone method (6). Glyoxylic acid, standardized acidimetrically, was used as a standard. Glycine-NaOH buffer without cytochrome c served as a control. In the calculations, the small extent of reduction and glyoxylic acid at zero time were subtracted from the results.

Dinitrophenylhydrazone of the product of glycine was prepared from a similar experiment as described by Cavallini et al. (7). Metaphosphoric acid was used for deproteinization. Thin layer chromatography of the product, together with the dinitrophenylhydrazone of glyoxylic acid was performed in butanol-ethanol-H_2O 50:10:40 (8).

All materials employed were of analytical grade. The glycine used in most of the experiments was a product of Fluka. In a comparative study, Merck and BDH glycine was tested. All the solutions were prepared in double distilled water.

RESULTS

In Fig. 1 the 550 nm absorbancy of 6×10^{-5} M ferricytochrome c in 0.2 M solutions of various buffers, pH 9.5, 37°C, is plotted as a function of time. The highest extent of reduction was obtained with glycine, followed by DL-alanine and glycyl-glycine. In DL-valine and triglycine, only a slight reduction was observed, while incubation in Tris-HCl, N-acetyl glycine and carbonate-bicarbonate buffers resulted in no reduction at all for time up to 6 hours.

The initial rate of the reduction of ferricytochrome c increased linearly with the concentration of glycine (Fig. 2). The above evidence

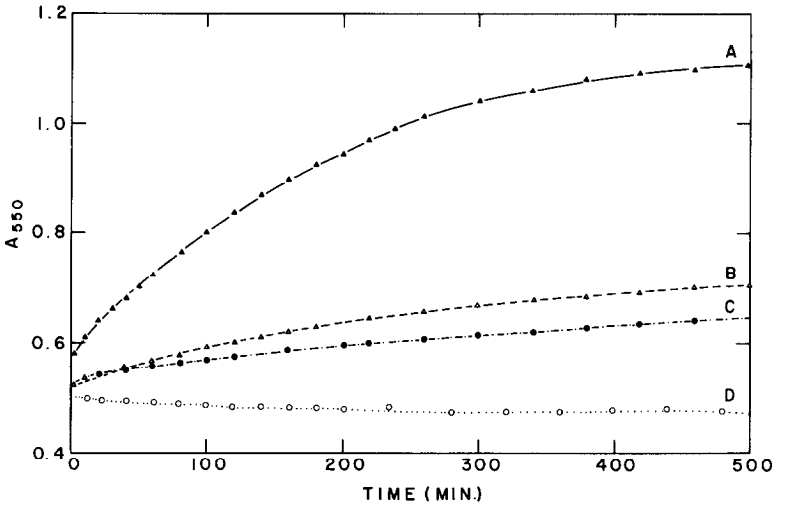

Figure 1 The time course of the reduction of 6 x 10^{-5} M ferricytochrome c in 0.2 M, pH 9.5 buffers. A. glycine-NaOH. B. glycyl-glycine-NaOH. C. DL-alanine-NaOH. D. Tris-HCl. 37°C.

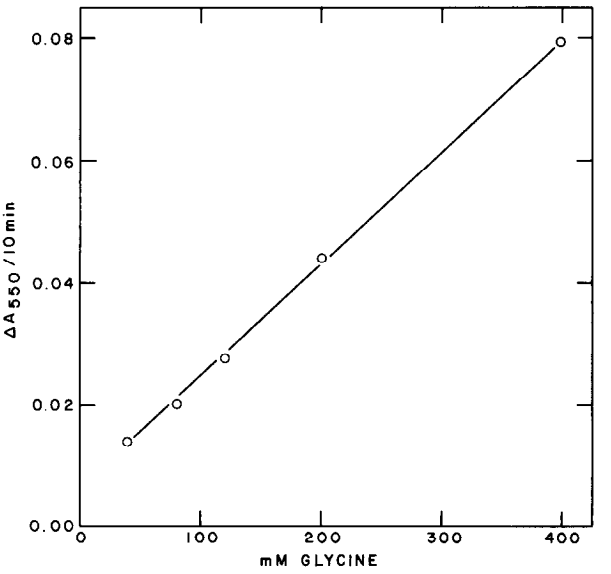

Figure 2 Initial rates of 6 x 10^{-5} M ferricytochrome c reduction at pH 9.5 as a function of the concentration of glycine. 37°C.

suggested that the reduction of cytochrome c in glycine buffer was due to a reducing agent present in the buffer itself. To check for a possible impurity, glycines manufactured by different companies were compared. Though slight differences were found, the reduction effect was similar. Furthermore,

repeated recrystallizations of glycine did not affect its reducing power. The most likely explanation for the effects of glycine would be therefore a direct reduction of cytochrome c by the amino acid with simultaneous formation of glyoxylic acid. It is known that glycine is oxidized by D-amino acid oxidase in the liver and kidney of mammals (9).

Attempts were made to identify glyoxylic acid in the incubation mixture. Both the pyrogallol (5) and phenylhydrazone (6) methods detected glyoxylic acid as a product of the reduction of ferricytochrome c with glycine, the latter method being more sensitive. No glyoxylate was formed upon incubation of glycine-NaOH in the absence of cytochrome c. In Fig. 3 the simultaneous appearance of both ferrocytochrome c and glyoxylic acid is plotted against time. The stoichiometry of the reaction is 1:2, namely, for one μmole of glyoxylic acid formed, two μmoles of ferricytochrome c undergo reduction.

The chromatographic migration of the dinitrophenylhydrazone derivative of the reaction product was identical to that of the dinitrophenylhydrazone of glyoxylic acid.

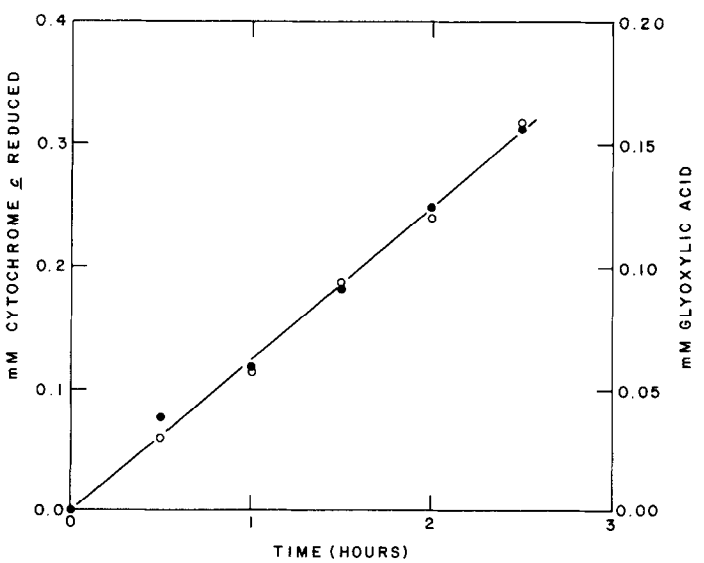

Figure 3 Ferricytochrome c (7×10^{-4} M) reduction (●) and glyoxylic acid formation (o) in 0.25 M glycine-NaOH buffer, 37°C. pH 9.5.

DISCUSSION

The experiments described above demonstrate that the reduction of cytochrome c incubated in glycine-NaOH buffer results from a direct transfer of reducing equivalents from the amino acid to the heme protein, accompanied by the formation of glyoxylate. This should require a 1:2 stoichiometry, as defined by equation (1):

(1) $2\ \text{cyt}^{+3} + \text{glycine} + H_2O \rightleftharpoons 2\ \text{cyt c}^{+2} + \text{glyoxylic acid} + NH_3 + 2H^+$

The experimental results are in keeping with this requirement. (Fig. 3).

The deamination of glycine leading to the formation of glyoxylate is a well known metabolic pathway (9). Furthermore, certain bacterial enzymes were shown to catalyse the reduction of cytochrome c by glycine and the conversion of the latter to glyoxylic acid (10). In the present study, a similar, nonenzymic reaction is described. The finding that the rate of reduction has a pH optimum of 9.9 (2) was confirmed in our experiments. The fact that the amino group of glycine is directly involved in the reaction suggests that the increment of the rate with pH is due to the increasing proportion of unprotonated glycine. The decrease above pH 9.9 indicates that a residue in the cytochrome c protein must undergo an ionization that prevents the reaction between cytochrome c and glycine. Since the apparent dissociation constant of the amino group of glycine is 9.6 (11) the pK of the protein residue may be estimated as 10.3, suggesting the involvement of either tyrosine or lysine in the reaction. Work, now in progress, employing modified forms of cytochrome c may help to identify the residue involved.

The rate of the reduction at pH 9.5 decreases when glycine dimer is substituted for its monomer and, in the case of the trimer, the reaction is very slow. The pK of glycyl-glycine is 8.17 (11); thus, when compared to glycine, the reaction at pH 9.5 (Fig. 1) should be more favorable, other things being equal. Steric hindrance seems to be the most plausible explanation for the lower rate obtained with the glycine peptides. One could expect other amino acids to interact

with cytochrome c in a similar way. In fact, DL-alanine and DL-valine were effective, but the reduction was slow, especially with the latter, in keeping with the postulated steric restrictions.

In conclusion, the concept of autoreduction is not applicable to experiments performed in solutions containing glycine and possibly other amino acids. It can be speculated that the blocking of the amino terminal glycyl residue of all vertebrate cytochromes c (12) has the function of preventing the oxidized enzyme from undergoing intramolecular dismutation leading to the ferrous state. Since the expected pK of amino terminal group in polypeptides and proteins is 7.8 (11), even at neutral pH the rate of autoreduction could be significant, especially if the terminal group is located near the site on the molecule that can accept reducing equivalents from glycine. In all non-vertebrate eukaryotic organisms, the same protection against autoreduction should be achieved by the elongation of the amino-terminal sequence, (12), which causes the removal of the N-terminal group from the electron accepting site.

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