REVERSAL OF THE ALKYLATION OF THE METHIONINE RESIDUES OF CYTOCHROME *c*

Abel SCHEJTER and Irit AVIRAM
Department of Biochemistry, Tel-Aviv University, Ramat-Aviv, Israel

Received 3 February 1972

1. Introduction

In the presence of cyanide, horse cytochrome *c* is alkylated by bromoacetate at the methionyl residues in positions 65 and 80; the nature of the reaction and the properties of the modified protein have been described elsewhere [1]. It has also been demonstrated [2] that iodoacetamide and iodoacetic acid react with cytochrome *c* in a similar way. During a series of studies on cytochrome *c* modification, it was of interest to investigate the possibility of reversal of the alkylation of the methionyl residues. In this communication we describe the reappearance of biologically active cytochrome *c* upon treatment with cysteine of cytochrome *c* previously alkylated by iodoacetamide.

2. Materials and methods

Horse heart cytochrome *c* (Sigma, Type III), purified on Amberlite CG-50 [3], was reacted with freshly recrystallized iodoacetamide (50 mg/ml) in the presence of 0.1 M KCN at pH 7.0 [1]. In experiments with radioactive material, 25 μCi of ^{14}C-iodoacetamide (Radioactive Center, Amersham) were added per ml of the reaction mixture. After 24 hr the mixture was diluted and passed through an Amberlite CG-50 column [3] in order to remove the reagents; the adsorbed protein was eluted, after repeated washings, with 0.3 M NaCl, dialyzed and lyophilized.

Radioactivity measurements were performed on a Packard Tri-Carb scintillation counter. Spectra were recorded on a Cary 15.

The oxidation of cytochrome *c* by oxygen in the presence of cytochrome oxidase, prepared according to Yonetani [4], was determined polarographically in the presence of ascorbate using an oxygen electrode [5].

3. Results

The reversal of the alkylation of the methionyl residues of cytochrome *c* by cysteine can be followed by measuring either the protein-bound radioactivity of the enzyme modified with a ^{14}C-labelled reagent or by determining the radioactivity released into the medium. In addition to that, the reversal of the alkylation of the methionyl 80 residue can be followed by observing the appearance of the 695 nm band, typical of the native enzyme in its oxidized state [6] and by testing the reducibility of cytochrome *c* in the reaction mixture. This is so because cysteine is unable to reduce cytochrome *c* alkylated at the methionine 80 [1].

In the first method, 3×10^{-5} M cytochrome *c* modified with iodoacetamide, was incubated with cysteine at pH's 7.0, 8.0 and 9.5, at 37°. Aliquots of 0.6 ml were removed at several times, added to 0.15 ml 30% trichloroacetic acid and centrifuged. The precipitates were washed and dissolved and their radioactivities as well as the radioactivities of the supernatants were counted. The results obtained at pH 8.0 and 75 mM cysteine show (fig. 1) that as the reaction proceeds the protein bound radioactivity decreases and appears in the medium. After 20 hr about 15% of the total radioactivity remains bound to the protein and is not removed by prolonged incubation. This is probably due to a partial modification of histidine 33

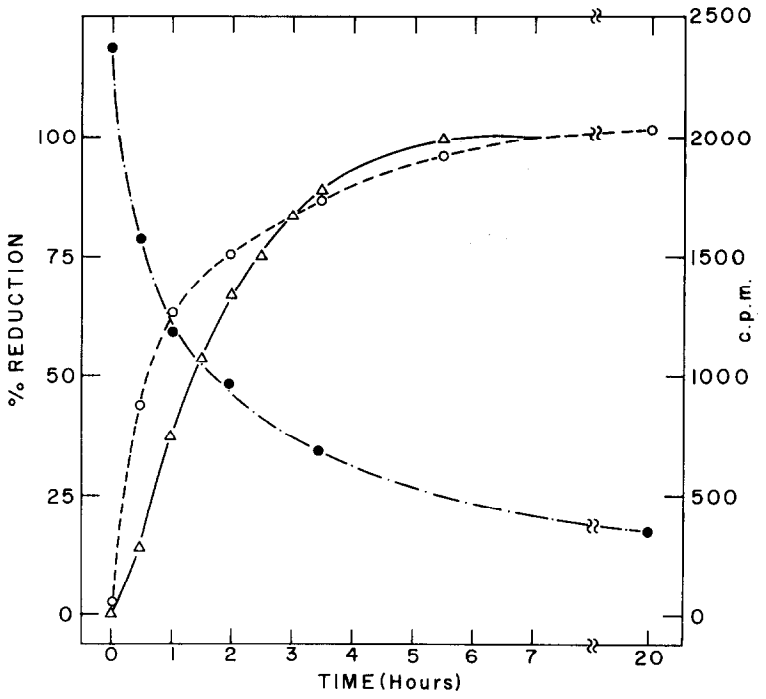

Fig. 1. Time course of the dealkylation of 3×10^{-5} M modified cytochrome *c* in the presence of 75 mM cysteine, pH 8.0, 37°. (●–●–●): Protein bound radioactivity; (○–○–○): radioactivity in the TCA supernatants; (△–△–△): reducibility by cysteine followed at 550 nm.

[2] which does not transfer its alkylating group to cysteine. The rate of the reaction depends on pH, as shown in fig. 2. The times needed for removal of 50% of potentially removable radioactivity under the conditions of the experiment at pH 7.0, 8.0 and 9.5 were 78, 40 and 18 min, respectively. It should be noticed that even in the absence of cysteine the removal of protein bound radioactivity was noticeable (fig. 2). The rate of this spontaneous splitting of the $-CH_2CONH_2$ group increased also with pH. At 37° and pH 9.5 about one third of the radioactivity appeared in the supernatants after 20 hr. At constant pH (8.0) and temperature (37°) the rate increased with the concentration of cysteine, half times of the reaction being 40, 52 and 87 min for 75, 50 and 25 mM cysteine, respectively. These results are consistent with a bimolecular reaction mechanism.

In a similar experiment at pH 8.0, 37° and 75 mM cysteine, the extent of reduction of the enzyme was followed at 550 nm. The results, expressed as percent of reduced cytochrome *c*, are shown in fig. 1. The reaction thus observed is slower at the beginning than the release of radioactivity into the medium; later it becomes faster, and reaches completion earlier.

After 20 hr, the reacted material was purified from the reagents on a Sephadex G-25 column. Upon oxidation with ferricyanide, the 695 nm band could be clearly observed.

The ability of the product of the cysteine treatment to mediate electron transfer from ascorbate to oxygen in the presence of soluble cytochrome oxidase was identical to that of native cytochrome *c* (table 1).

4. Discussion

The experiments described above show that cysteine can effect the reversal of the alkylation of the methionyl residues of cytochrome *c* by iodoacetamide, leading to the recovery of the enzyme with its original native properties, namely, enzymic activity in the cytochrome oxidase system, reducibility with cysteine

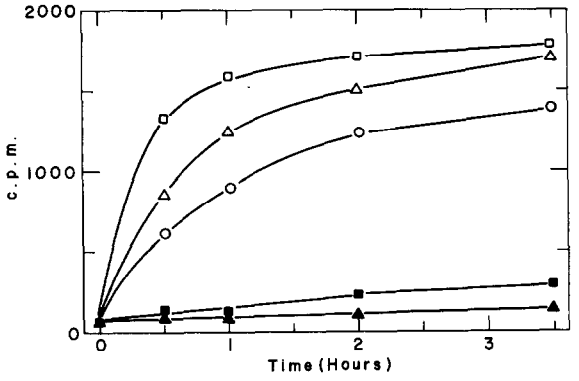

Fig. 2. The effect of pH on the dealkylation of modified cytochrome *c* in the presence and absence of 75 mM cysteine; 37°. (□–□–□): pH 9.5, cysteine; (△–△–△): pH 8.0, cysteine; (○–○–○): pH 7.0, cysteine; (■–■–■): pH 9.5, no cysteine; (▲–▲–▲): pH 8.0, no cysteine.

Table 1
Catalytic activity of cytochrome *c* in the ascorbate–cytochrome oxidase system [5].

Cytochrome *c*	Oxygen uptake (nmoles/min)
Native	36
Alkylated	2
Dealkylated	39

3×10^{-5} M cytochrome *c*, 33 mM ascorbate, 0.05 M phosphate buffer pH 7.0, 5×10^{-8} M cytochrome oxidase. Total vol = 3 ml.

and ascorbate and existence of the 695 nm band in the oxidized state [6].

Transfer of a methyl group from a sulfonium salt to a sulfhydryl acceptor is a well-known reaction [7]. Hence, it is not surprising to find that this can be an effective method for the removal of other alkylating groups from proteins thus modified at their methionine sulfurs. Nevertheless, it should be pointed out that in the case of proteins the conditions for optimal reactivity of alkylated methionine sulfurs may change from one case to another. In general, it should be expected that increase in pH, temperature and concentration of the acceptor should increase the rate of the removal, as in the case of cytochrome *c* described here.

It should be noted that even in the absence of acceptor the alkylating group is slowly removed (fig. 2). Since the rate of this removal increases with pH, it is most probable that it is due to nucleophilic attack by hydroxyl ions.

One observation deserves comment. As seen in fig. 1, the removal of the radioactivity follows an apparent first order kinetics; on the other hand, the rate of reappearance of cysteine reducible cytochrome *c* is slower at the beginning and increases later, resulting in a sigmoid time dependence. It is obvious from this comparison that the dealkylation of the methionyl 65 residue proceeds faster than that of the methionyl 80 residue which is responsible, when coordinated to the iron, for the typical properties of native cytochrome *c*. Such a difference may result from steric hindrance for the access of cysteine to the methionyl 80 residue, or from the existence of a different mechanism for the dealkylation of the two residues.

Work is now in progress to elucidate the mechanism of the dealkylation reaction and to establish its characteristics in other proteins and with other alkylating groups.

References

[1] A. Schejter and I. Aviram, J. Biol. Chem. 245 (1969) 1552.

[2] K. Ando, H. Matsubara and K. Okunuki, Biochim. Biophys. Acta 118 (1966) 240.
[3] E. Margoliash and O.F. Walasek, in: Methods in Enzymology, Vol. X, eds. R.W. Estabrook and M.E. Pullman (Academic Press, New York, 1967) p. 339.
[4] T. Yonetani, in: Biochemical Preparations, Vol. 11, ed. A.C. Maehly (Wiley, New York, 1966) p. 14.
[5] L. Smith and P.W. Camerino, Biochemistry 2 (1963) 1428.
[6] A. Schejter and P. George, Biochemistry 3 (1967) 1045.
[7] G.L. Cantoni, in: Comparative Biochemistry, Vol. 1, eds. M. Florkin and H.S. Mason (Academic Press, New York, 1960) p. 181.