ACID AND ALKALI DENATURATION OF RHODOSPIRILLUM HAEM PROTEIN

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(Received June 9th, 1961)

SUMMARY

1. Exposure of Rhodospirillum haem protein to pH 12.5 for 15 min results in the irreversible denaturation of some of the protein. This denaturation is more pronounced with an increase in exposure time; after 5 h the protein cannot be "regenerated".

2. The only detectable effect consequent on exposure of the protein to pH 1.9 for 15 min is the formation of aggregates.

INTRODUCTION

RHP has been shown to be a naturally occurring protein[1,2]; among other possibilities, it can function as an activator of photophosphorylation[3].

The spectro-chemical properties of RHP have been the basis for the conclusion that this haem protein is a variant form of cytochrome *c*. Thus, RHP at neutral pH exhibits an acid hematin spectrum, similar to that of myoglobin while, at pH $>$ 11, a true cytochrome *c* spectrum is obtained. Within the limitations imposed by the spectrophotometric assay methods used, this change is completely reversible; the original acid hematin-like spectrum reappears when the pH is lowered to 7.0.

The possibility that exposure of RHP to a cycle of pH changes results in irreversible denaturation remains in spite of the apparent reversibility of spectral changes, because changes in protein structure may occur which are not effective in prevention of reconstitution of the environment around the haem iron. This question can be investigated by a combination of immunochemical[2] and spectrochemical assays developed in recent researches. The results of such investigations are presented in this paper.

METHODS

Crystalline RHP as prepared by HORIO AND KAMEN[4] was kindly supplied by Dr. HORIO. All spectrophotometric determinations were made with a Cary Model 14 recording spectrophotometer. Sedimentation velocities were determined in the usual

Abbreviation: RHP, Rhodospirillum haem protein.

* Paper No. 130 in the series "Communications of the Graduate Department of Biochemistry, Brandeis University, Waltham, Mass. (U.S.A.)".

manner with the Spinco Model E analytical ultracentrifuge. For immunochemical analysis of RHP the complement fixation method, described in a previous communication[2], was employed.

RESULTS AND DISCUSSION

Spectroscopic observations

0.207 mg/ml of RHP (approx. $7.4 \cdot 10^{-3}$ μmoles/ml) in $M/15$ phosphate buffer, pH 7.0, was divided into three equal aliquots. One portion was kept at pH 7.0; the remaining two samples were brought to pH 1.9 and 12.5 with 10 N H_3PO_4 and 10 N KOH respectively. The reagents were delivered by means of a micro-pipet so that volume changes were negligible. After 15 min of exposure to these pH conditions at room temperature, the pH was returned to 7.0 and the absorption spectra were recorded.

Oxidized and reduced spectra are reproduced in Fig. 1. The spectra in the visible region are superimposible. However, acid and alkali treated RHP upon reduction exhibit small changes in the intensity of the Soret maximum at 424 mμ and of the shoulder at 431 mμ. Some deviation from the spectrum of the oxidized form is also evident with acid and alkali treated RHP in the far ultraviolet region. It is evident that the spectroscopic characteristics of RHP are affected but little by exposure of the protein to these extremes of pH for 15 min.

However, when RHP is kept at pH 12.5 for 5 h and the pH then returned to 7.0, there is no reappearance of the characteristic acid hematin spectrum but the spectrum of a typical cytochrome *c*-like haemochromogen persists (Fig. 2). These results show that RHP is denatured when exposed for long periods to alkali but is only slightly affected by the same treatment for short periods. Moreover, the absorbancy of the oxidized Soret peak is higher than that of the reduced Soret peak, rather than lower.

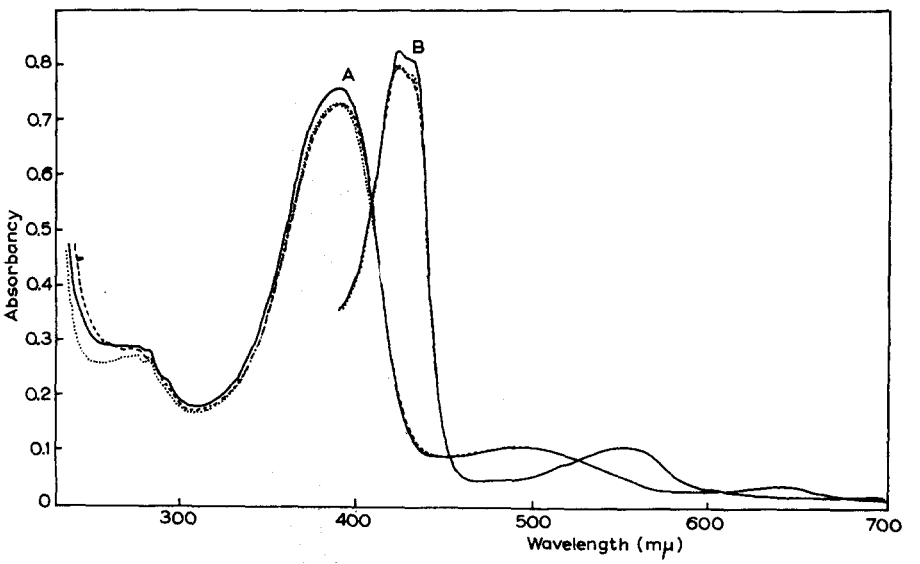

Fig. 1. The effect of acid and alkaline pH on the absorption spectra of RHP. Native RHP, ———, was subjected to acid, ·····, and alkali, – – – –, as described in the text. The curves indicated by (A) represent the oxidized forms and (B) dithionite reduced RHP.

Immunochemical analysis

To determine whether the treatments described above effected changes in RHP which could not be detected spectroscopically, the samples have been examined serologically. It has been shown in a previous communication[2] that the serological properties of RHP are dependent upon the degree of denaturation; thus, extensively denatured RHP produces no reaction with antiserum directed against native protein.

The serological titration curves of acid treated, alkali treated and native RHP are shown in Fig. 3. For samples exposed for 15 min, acid treated RHP exhibits serological behavior which is identical with that of the native form. The titration curve of alkali treated RHP, on the other hand, is markedly different from that of the native form in the region of antibody excess and peak fixation is displaced to higher concentrations of RHP; thus, $0.8 \cdot 10^{-3}$ μg N/ml is required for peak fixation with native RHP while twice this amount, $1.5 \cdot 10^{-3}$ μg N/ml, produces peak fixation with alkali treated RHP. The requirement for twice the amount of antigen to give comparable fixation as the

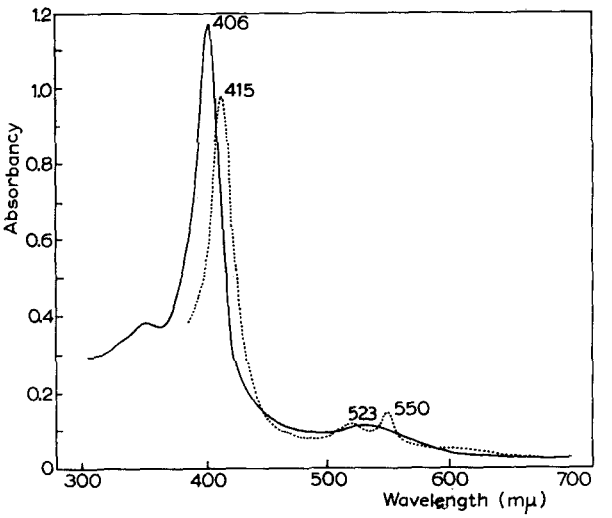

Fig. 2. The absorption spectra at pH 7.0 of RHP exposed to pH 12.5 for 5 h. ———, Oxidized RHP; . . . . . , reduced RHP.

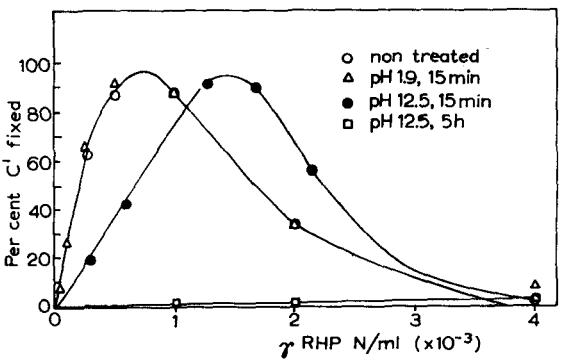

Fig. 3. The immune reactivity of RHP exposed to acid and alkali. Conditions are described in the text.

homologous native protein may be taken to mean that the antigen is a mixture of native and irreversibly denatured RHP.

The denaturation of proteins by alkali is well known and theoretical aspects have been presented in detail by KAUZMANN[8]. Thus, the addition of alkali to RHP to a pH far beyond the isoelectric point[4] (pH 4.6) is accompanied by dissolution of secondary and tertiary bonds and subsequent unfolding of peptide chains. This unfolding of RHP can be correlated with the disappearance of the acid hematin spectrum and appearance of the haemochromogen spectrum. Neutralization to pH 7.0, if accomplished before an exorbitant amount of such unfolding occurs, causes regeneration of native RHP by re-establishment of the original environment. However, reversal of denaturation produces conformations of RHP not wholly identical with the native form. Thus, RHP, denatured by alkaline treatment for 15 min, shows a serological specificity different from that of the native protein. At least 50% of the RHP so treated is altered to such an extent that the antigen–antibody reaction is diminished or lost.

It is noteworthy that this extensive denaturation is not evident from the absorption spectra of Fig. 1. The absence of any marked changes in the original spectrum attendant upon alkaline denaturation and "regeneration" of RHP can be understood on the basis that the original bonding is re-established in the vicinity of the haem sites responsible for the characteristic RHP spectrum.

The irreversible denaturation of RHP by alkali becomes more pronounced as the exposure time is lengthened and after 5 h at pH 12.5 there is complete loss of serological reactivity. These facts indicate inability of the unfolded peptide chains to reconstitute the environment around the haem sites after extensive unfolding.

Ultracentrifugation

To examine further the physical state of RHP after varied exposure to extremes of pH the sedimentation behavior of samples of RHP, treated as above, were studied. During an experiment, which involved sedimentation of 1 h duration at 59780 rev./min in a standard cell with a Kel F centerpiece, no sedimentation of colored material, or formation of a peak, was noted in the sample which had been treated for 5 h at pH 12.5. Since it was not likely that RHP had been fragmented into peptides under such comparatively mild conditions, it was not expected that the absence of any sedimentation would occur. It was concluded that the unfolding of proteins in alkaline solution may be accompanied by a considerable increase in viscosity, an effect well known from other studies[5]. An increase in viscosity, in turn, could have prevented sedimentation.

With each of the RHP samples obtained by short-term exposure (15 min) a peak

TABLE I

COMPARISON OF THE SEDIMENTATION VELOCITIES AND MOLECULAR WEIGHTS OF RHP EXPOSED TO ACID AND ALKALINE pH FOR 15 MIN

Conditions	s_{20}	*Molecular weight*
Native	2.74	27 300
Acid treated	3.12	31 100
Alkali treated	2.45	24 400

was formed at the gradient of the refractive index which coincided with the colored boundary. The sedimentation coefficients and molecular weights, shown in Table I, were calculated according to procedures described by SCHACHMAN[6]; the differences are well beyond variations attributable to experimental error. The values, s_{20} of 2.74 and molecular weight 27300, for native RHP are identical with those reported previously by HORIO AND KAMEN[4].

The sedimentation pattern of acid treated RHP was characterized by a rather broad peak and some trailing of the base line suggestive of changes in the degree of homogeneity, owing apparently to the formation of some aggregated RHP. The average molecular weight was larger than that of native RHP.

While RHP appears to be relatively stable to acid conditions, it is precipitated at low pH, and so aggregation is not unexpected. The uniform serological behavior of acid treated RHP (Fig. 3) is like that exhibited by aggregates of denatured egg albumin[7]. Thus, aggregates of acid denatured egg albumin are serologically identical with homologous protein in the region of antibody excess; whereas more nitrogen is precipitated by denatured egg albumin in the region of antigen excess. It is possible to relate the degree of aggregation to the amount of nitrogen precipitated which is increased in the antigen excess zone by highly aggregated protein. The coincidence of the immune titration curves of acid treated RHP and the native form suggests that a low degree of aggregation exists which is beyond the limits of detection by this method.

The sedimentation velocity and molecular weight of alkali treated RHP are found to be less than those of the native form. This effect can be ascribed probably to differences in shape and hydration between the native form and denatured RHP.

ACKNOWLEDGEMENTS

The author is indebted to Professor M. D. KAMEN, Drs. T. HORIO, L. LEVINE and M. SOODAK for many helpful discussions and assistance in the preparation of this manuscript.

The author is a Postdoctoral Research Fellow of the United States Public Health Service. These researches were supported by grants-in-aid from the National Institutes of Health (C3649-4) and the National Science Foundation (G-6441).

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