A Spectrophotometric and Fluorimetric Study of Alkaline Transitions of *Euglena* Cytochrome *c* 552†

Irit Aviram,* Graham W. Pettigrew,‡ and Abel Schejter

ABSTRACT: The behavior of the photosynthetic cytochrome *c* 552 upon titration with alkali depends on the ionic composition of the medium. In water the disappearance of the 695-nm band, indicating the displacement of the methionine ligand, as well as a remarkable tryptophan fluorescence enhancement, follow a single proton titration curve with pK of 10.0 and n = 1.0. The product is a low spin type protein. In salt-containing media two successive steps are observed: in the first one, completed at about pH 10.3, a high-spin form of cytochrome *c* 552 is obtained and relatively small fluorescence enhancement is detected. In the second step, more profound fluorometric changes occur, while the material reverts to its low-spin form. Addition of salts to an alkaline solution of cytochrome *c* 552 in water results in the formation of a 600-nm high-spin band with a concomitant quenching of tryptophan fluorescence. The results imply that at high pH unfolding of the molecule is evident only when the low-spin product is obtained. In the high-spin alkaline form, the methionine ligand is probably displaced from iron coordination by hydroxyl ions, while in the low-spin alkaline form methionine may be replaced by a lysyl residue of the cytochrome *c* 552 protein. The results imply that the lysyl residue is available for coordination in salt solutions at a higher pH than in water.

In the empirical classification of the cytochromes based on their spectral properties, the cytochrome *c* class includes those cytochromes for which the α band of the reduced state is found at or near 550 nm. Structurally, the *c*-type cytochromes have in common the covalent attachment of their prosthetic group to the protein through one or two thioether bonds. The photosynthetic cytochrome *c* 552 of *Euglena gracilis* belongs to the cytochrome *c* class. Its prosthetic group is covalently attached to the protein (Pettigrew, 1974); its spectrum and heme-linked ionizations (Ben Hayyim and Schejter, 1974) are similar to those of other members of this class. Most probably histidine and methionine residues are coordinated to the iron (Ben Hayyim and Schejter, 1974), the latter linkage being responsible for the 695-nm band in the native conformation at neutral pH (Shechter and Saludjian, 1967).

Spectrophotometric studies of mammalian cytochrome *c* (Davis et al., 1974) and the bacterial cytochrome c_2 of *Rodospirillum rubrum* (Pettigrew and Schejter, 1974) correlated the disappearance of this band at high pH with an ionization dependent conformation change in the molecule. Participation of a lysine residue replacing methionine in the above transition was implied (Davis et al., 1974; Wilgus and Stellwagen, 1974).

Changes in spectra of hemoproteins could arise, however, from ligand exchange involving only small perturbations in the vicinity of the prosthetic group. An additional and independent criterion is required to confirm the conformation change hypothesis. This was found in the case of *Euglena* cytochrome *c* 552 by use of tryptophan fluorescence measurements. This report summarizes fluorimetric and spectrophotometric studies of the alkaline transition of cytochrome *c* 552 and its dependence on the ionic composition of the medium.

Experimental Procedure

Cytochrome *c* 552 was prepared as described by Pettigrew (1974). It was oxidized prior to use with a small excess of ferricyanide and used without purification in some of the spectrophotometric measurements in the visible range. For direct measurements in the Soret region and for fluorimetry, the excess of the reagents was removed on a G-25 Sephadex column. The same procedure was employed when ascorbate reduced cytochrome *c* 552 was tested.

Spectrophotometric titrations were performed on a Cary 118 spectrophotometer, by titrating with 0.2–2.0 N NaOH in the cuvette with a microsyringe. The total volume of alkali added to 2.5 ml of protein solution never exceeded 25 μl. The pH was measured on a Radiometer pH meter 26. In titrations with salts at alkaline pH, a sample of cytochrome *c* 552 in water at a desired pH was titrated with an equimolar solution of the protein in 4 M salt, adjusted to the same pH.

Fluorescence was measured on a Hitachi Perkin Elmer spectrofluorimeter on 10^{-6} M solutions of cytochrome *c* 552. At this concentration range fluorescence was proportional to concentration. Titrations were performed as described above. Changes in the absorption in the excitation and emission range during titration were negligible compared to changes in fluorescence. A 2 μM solution of *N*-acetyl-L-tryptophanamide (Cyclo Chemical) served as standard for comparison of relative fluorescence yields.

Results

Ben Hayyim and Schejter (1974) reported that cytochrome *c* 552 undergoes an alkaline ionization leading to the disappearance of the 695-nm band. Their titrations, performed in the Soret region in 50 mM buffers, permitted estimation of a pK of 9.4 for this transition and a Hill coefficient of 1.4. On the basis of the latter result the authors

† From the Department of Biochemistry, The George S. Wise Center for Life Sciences, Tel Aviv University, Tel-Aviv, Israel. *Received August 15, 1975.* This work was supported by a grant from the United States–Israel Binational Science Foundation (BSF), Jerusalem, Israel.

‡ Exchange Fellow, Royal Society of London–Israel Academy. Present address: Department of Chemistry, University of California—San Diego, La Jolla, California 92037.

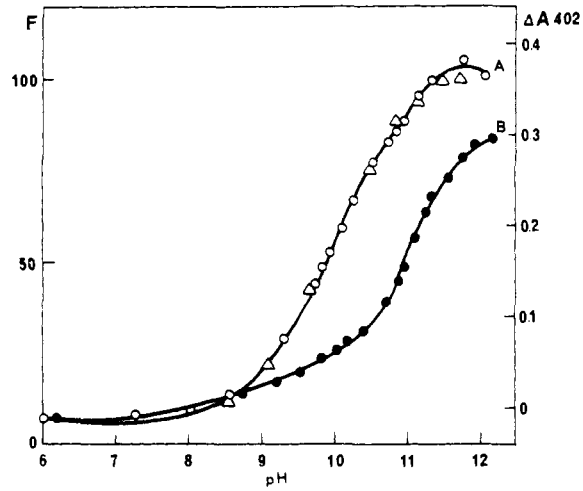

FIGURE 1: Spectrophotometric (△) and fluorimetric (●,○) titrations of cytochrome *c* 552: (A) in water, (B) in the presence of 0.5 M NaCl. Spectrophotometric measurements were made by recording difference spectra at the Soret peak on a 1.2×10^{-5} M cytochrome *c* 552 solution in water. Fluorescence intensity was determined at 350 nm on a 10^{-6} M solution of the protein. Excitation wavelength, 290 nm.

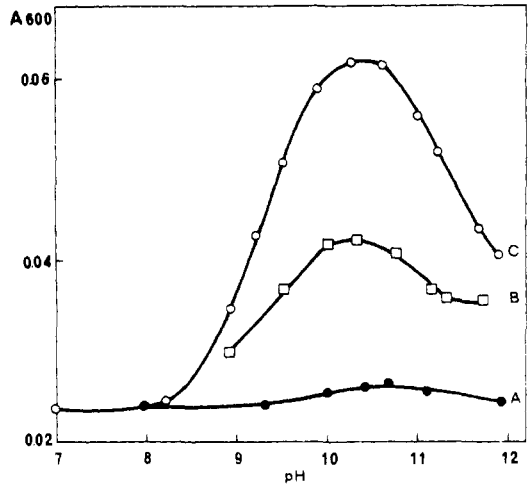

FIGURE 2: Spectrophotometric titration at 600 nm of a 1.70×10^{-5} M solution of cytochrome *c* 552: (A) in water, (B) in 0.1 M NaCl, (C) in 0.5 M NaCl.

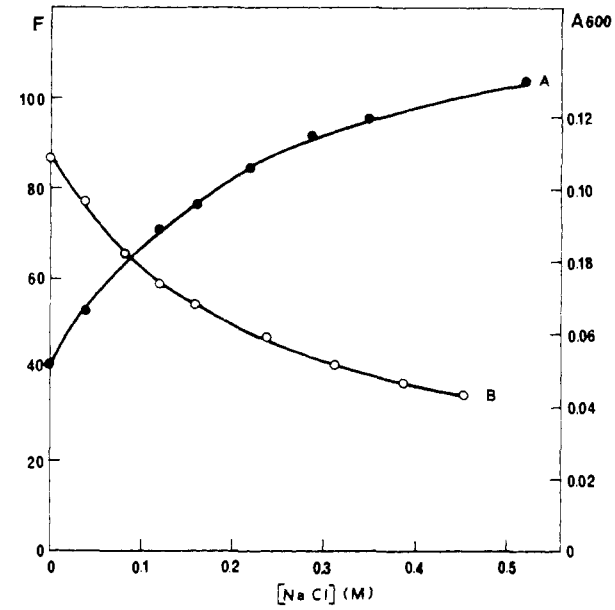

FIGURE 3: Spectrophotometric (A) and fluorescence (B) titration of an aqueous solution of cytochrome *c* 552 with NaCl. (Concentrations were: 3.4×10^{-5} in A and 10^{-6} M in B. Fluorescence was measured as described in Figure 1).

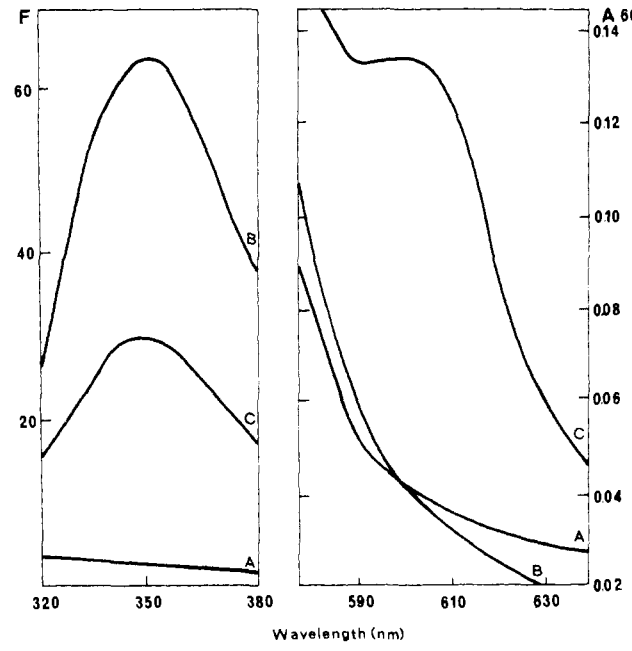

FIGURE 4: Fluorescence emission and optical absorption spectra of cytochrome *c* 552: (A) in water at pH 6.0, (B) in water at pH 11.2, (C) pH 11.2 in 0.4 M NaCl.

concluded that the process involved more than one proton ionization. In this regard, cytochrome *c* 552 apparently differed from mammalian cytochrome *c*, which shows a Hill coefficient of 1.0 for the same ionization (Davis et al., 1974).

Upon further investigation of this result it was found, in the present study, that the spectral changes occurring upon alkalinization of cytochrome *c* 552 solutions depend on the salt concentration of the medium. In water, the disappearance of the 695-nm band is accompanied by a decrease and red shift of the 526-nm peak and increase and blue shift of the Soret peak. The spectra have well-defined isosbestic points and an identical p*K* of 10 at each wavelength with $n = 1$ (Figure 1).

More complex spectral changes appear when titrations are performed in the presence of salts. At 600 nm a shoulder appears around pH 9.0 indicating the presence of a high-spin form. The shoulder intensifies up to pH 10.3 and decreases in intensity upon further raise in pH (Figure 2). Similarly, absorbance of the 526-nm peak decreases up to pH 10.3 and increases thereafter. In the Soret difference spectra isosbesticity is lost above pH 10.3. When sufficiently concentrated salt solutions, such as 0.5 M NaCl, are titrated the absorbance at 695 nm reaches its minimum above pH 9, although the shoulder disappears only at higher pH. Here the disappearance of the 695-nm band is masked by an increase in absorbance in this range. These results indicate that in alkali, in the presence of salts, cytochrome *c* 552 undergoes two consecutive changes: in the first stage, completed at about pH 10.3, a high-spin form of the protein is formed, followed by its conversion to a low-spin spectral type at higher pH. None of these phenomena

are observed in the case of mammalian cytochrome *c*.

The fraction of the high-spin form at a given pH increases with the concentration of the salt. This dependence is represented by a titration with salts shown in Figures 3 and 4. Saturation of the effect is observed at about 0.8 M NaCl. KCl and CsCl have similar effect, Na_2SO_4 is more effective, while sodium perchlorate, recognized for its disrupting action on macromolecular structure, causes a less pronounced increase of the high-spin band. These changes are reversible: when the alkaline solution is passed through a Sephadex G-25 column, the neutral, salt-free material reverts to the low-spin spectral type.

The yield of fluorescence of aromatic residues of hemoproteins is very low, due to a nonradiative energy transfer from these residues to the prosthetic group (Weber and Teale, 1959). This precludes the use of this sensitive tool in the investigation of native hemoproteins, including mammalian cytochrome *c*. Similarly, the fluorescence of the two tryptophans of neutral cytochrome *c* 552 is quenched. However, upon alkalinization of an aqueous solution of the cytochrome a very pronounced enhancement of the tryptophan fluorescence at 350 nm is observed (Figure 4). A plot of intensity values vs. pH gives a typical titration curve with a pK of 10.0, $n = 1$ (Figure 1). The fluorescence and spectrophotometric titrations curves coincide. At pH 11, when the emission peak reaches its maximum, its intensity accounts for 50% of that of equimolar *N*-acetyl-L-tryptophanamide solution. Upon addition of NaCl to an aqueous solution at pH 11.0 the fluorescence is quenched as shown in Figure 3. This decrease could be related to the appearance of the high-spin band in the spectrophotometric measurements. When titration with alkali is conducted in the presence of NaCl (Figure 1), the relative intensities exhibit a gradual increase up to pH about 10 where more pronounced enhancement is detected. For this second stage a pK of about 11.2 can be evaluated. It should be noticed that when salt is added to an alkaline solution of cytochrome *c* 552 the resulting fluorescence quenching, as well as the 600-nm band intensity, are somewhat more pronounced than when continuous titrations are performed in the presence of the similar concentrations of salt at a similar pH (Figures 1 and 3).

These findings are characteristic of the oxidized form of cytochrome *c* 552. The reduced form, similarly to other cytochromes *c*, does not change upon addition of alkali. Fluorescence enhancement of ferrocytochrome *c* 552 in alkali does occur but amounts to only 27% of the respective enhancement in the oxidized enzyme. At least part of it can be attributed to autoxidation of cytochrome *c* 552 at high pH.

It is worth stressing that the fluorescence of the single tryptophan of mammalian cytochrome *c* is quenched both at neutral and at alkaline pH, with and without salt.

Discussion

The 695-nm band of *c*-type cytochromes in the ferric state is generally attributed to the native conformation of these proteins (Schejter and George, 1964), in which a methionine residue is bound to the iron. The disappearance of this band at alkaline pH is ascribed to an ionization dependent conformation change involving replacement of methionine by another protein group, presumably lysine (Davis et al., 1974; Wilgus and Stellwagen, 1974). Evidence presented so far for the conformation change was, however, indirect and related to the immediate environment of the heme.

Cytochrome *c* 552 belonging to the same group of cytochromes *c* undergoes a similar transition in alkali, with an observed pK of 10. The spectrophotometric titration curve in aqueous solution gives a Hill plot with $n = 1$, p$K = 10$ (Figure 1), and can be interpreted as a single proton dissociation corresponding to the alkaline ionization of horse cytochrome *c*, the product of which is a low-spin protein.

Concomitantly with spectral changes the tryptophan fluorescence undergoes a remarkable enhancement (Figures 1 and 4). In native cytochrome *c* 552 tryptophan fluorescence is quenched by energy transfer to the heme (Weber and Teale, 1959). Weber and Teale (1959) calculated for various hemoproteins the distance between heme and an aromatic residue at which the probability of energy transfer and emission are equal. For cytochrome *c* this distance was 30 Å, and indeed only in the denatured state the fluorescence of its tryptophan becomes unquenched (Fisher et al., 1973). A similar distance could be expected for cytochrome *c* 552. The enhancement of fluorescence encountered upon alkalinization could be thus caused either by unfolding of the molecule as a whole or at least of the tryptophan containing portion of it, and/or by a change of the angular position of tryptophan with respect to the heme group. Both possibilities require conformational transition involving a tryptophan residue which does not bind directly to the iron. Moreover any enhancement of the tryptophan fluorescence at alkaline pH, where amino groups and ionized tyrosine exert quenching effects, can be attributed to changes in conformation (Chien et al., 1969).

The good correspondence between fluorescence and absorption measurements (Figure 1) favors the hypothesis that the methionine displacement evidenced by loss of the 695-nm band is accompanied by a reorganization of the molecule. Experimental evidence of this kind cannot be obtained for mammalian cytochromes *c*, that have a single tryptophan whose fluorescence is quenched at neutral and alkaline pH.

Euglena cytochrome *c* 552 has two tryptophan residues (Pettigrew, 1974). The enhancement of its fluorescence could reflect an average effect on both residues or a more pronounced modification of the environment of one of them. In the different algal cytochromes of the *c* type that have been sequenced (Ambler and Bartsch, 1975) with which the *Euglena* cytochrome is homologous, one residue, tryptophan-59, is a conserved aromatic residue and occupies a position in relation to the proposed axial ligand, methionine-56, similar to the invariant phenylalanine-82 of mitochondrial cytochromes *c* (Dayhoff, 1972). The second tryptophan is near the C-terminus of the molecule (Pettigrew, 1974) and by analogy with the tertiary structure of cytochrome *c* (Takano et al., 1973) and cytochrome c_2 (Salemme et al., 1973) may be part of a terminal helical region. It would be of interest to examine the homologous cytochromes of this type which have phenylalanine in place of tryptophan-59 (Ambler and Bartsch, 1975) and also the *Monochrysis* cytochrome which contains no tryptophan at the C-terminus. The maximal fluorescence intensity at pH 11 corresponds to about 50% of the intensity of *N*-acetyltryptophanamide. In cytochrome *c* the fluorescence of the single tryptophan residue does not increase upon alkalinization. Thus if the latter possibility is considered, it appears that one of the tryptophans in *c* 552 is located closer to the surface of the molecule than the other. The finding that the emission band does not shift throughout the titration supports this suggestion. Partial unfolding of the molecule upon deprotonation of lysines may be due to electrostatic repulsion between the negative carboxyl groups on the pro-

tein. At pH 11, the only positive residue left is the single arginine (Pettigrew, 1974).

The behavior of cytochrome *c* 552 in the presence of salts poses difficulties of interpretation. Contrary to the usual alkaline transition which does not affect the low spin state of cytochromes *c*, including cytochrome *c* 552 in water, in media containing salt at concentrations above 20 mM the formation of a high-spin band was observed up to pH 10.3, followed by decrease of its intensity above this pH, indicating reversal to a low-spin product. Since the extinction of the intermediate form is unknown, Figure 2 permits only a very rough estimation of the p*K*'s of the two successive transitions: 9.3 and 11. It should be pointed out that cytochrome *c* 552 is the first *c*-type cytochrome reported to undergo a conversion to a high-spin or mixed-spin form around pH 9. Formation of a high-spin fraction has been reported for the respiratory cytochromes *c* 557 and *c* 558 (Pettigrew et al., 1975) but this took place at higher pH, after most of the 695-nm band was already lost. Spectral transitions identical with those shown above in the case of solutions of cytochrome *c* 552 containing salts have been demonstrated recently by Brittain and Greenwood (1975) for guanidinated horse cytochrome *c*. Since the p*K* of the guanido group is higher by 2 pH units than that of the amino groups of lysine, considered to bind heme iron at alkaline pH (Davis et al., 1974; Wilgus and Stellwagen, 1974), the authors proposed replacement of methionine by a hydroxyl group up to pH 10.5 with a subsequent decrease in spin upon coordination of the stronger ligand, deprotonated homoarginine. This explanation implies that the disruption of the iron–methionine bond at high pH is not due to competition with a lysine ϵ-amino group but rather results from weakening of the bond itself. Its replacement by lysine in the native protein is favored by a conformation change; but when an appropriate protein group is not available the solvent occupies the methionine binding site. Applying the same argument to the case of cytochrome *c* 552 in ionic medium, it appears that the particular lysine which binds the metal in water only, is unavailable for coordination up to about pH 10.3, either because of steric hindrance or elevated p*K*. Fluorescent measurements indicating that most of the conformation change occurs above the pH at which the first stage has been completed indicate that indeed unfolding is required for the formation of a low-spin species. Unfolding is repressed by salt as evidenced by the quenching effect of the latter, and higher pH is required to permit coordination by the lysine group. The elevation of the p*K* required for the formation of a low-spin species by the addition of salt is not likely to be related only to the shielding effect of the latter. The effect of salt increases up to concentrations higher than those necessary to saturate electrostatic repulsion (Figure 3). Specific binding could be involved.

One should remember, however, that the participation of a lysine residue in the alkaline transition of *c*-type cytochromes has not been proved directly. The spin state of iron depends on its position relative to the heme plane (Hoard, 1966); this, in turn, is determined not by the axial ligands only, but also by the protein conformation (Perutz, 1972). It has been suggested (Aviram and Krauss, 1974) that carboxymethylated cytochrome *c* can exist as a low-spin compound with the sixth coordination site vacant or occupied by a water molecule, provided that the conformation of the protein on the histidine side, through the histidine–iron bond, positions the metal in the porphyrin plane. In alkaline metmyoglobin and methemoglobin the binding of OH^- at the sixth coordination site results in a high-spin–low-spin equilibrium, the position of which differs for the different proteins (Antonini and Brunori, 1971). In view of this, the possibility that a hydroxyl ion replaces methionine in *c*-type cytochromes cannot be ruled out. In cytochromes so far examined at alkaline pH, including cytochrome *c* 552 in water, the resulting product would be low-spin due to conformational restrictions imposed upon the iron by the well-packed histidine side of the molecule. In cytochrome *c* 552 this portion of the molecule is affected by salts shifting the equilibrium in the direction of the high-spin form. Further increase in pH is required to restore the alkaline species to its low-spin form.

References

Ambler, R. P., and Bartsch, R. G. (1975), *Nature (London) 253,* 285.

Antonini, E., and Brunori, M. (1971), Hemoglobin and Myoglobin in their reactions with ligands, Neuberger, A., and Tatum, E. L., Ed., Amsterdam, North-Holland Publishing Co., p 52.

Aviram, I., and Krauss, Y. (1974), *J. Biol. Chem. 249,* 2575–2578.

Ben Hayyim, G., and Schejter, A. (1974), *Eur. J. Biochem. 40,* 569–573.

Brittain, T., and Greenwood, C. (1975), *Biochem. J. 147,* 175–177.

Chien, R. F., Edelhoch, H. E., and Steiner, R. F. (1969), in Physical principles and techniques of protein chemistry, Part A, Leach, S. J., Ed., New York, N.Y., Academic Press.

Davis, L. A., Schejter, A., and Hess, G. A. (1974), *J. Biol. Chem. 249,* 2624–2632.

Dayhoff, M. O. (1972), Atlas of Protein Sequence and Structure, Vol. 4, Washington, D.C., National Biomedical Research Foundation, pp D11–D27.

Fisher, W. R., Taniuchi, H., and Anfinsen, C. A. (1973), *J. Biol. Chem. 248,* 3188–3195.

Hoard, J. L. (1966), in Hemes and Hemoproteins, Chance, B., Estabrook, R. W., and Yonetani, T., New York, N.Y., Academic Press, pp 9–24.

Perutz, M. F. (1972), *Nature (London) 237,* 495–499.

Pettigrew, G. W. (1974), *Biochem. J. 139,* 449–457.

Pettigrew, G. W., Aviram, I., and Schejter, A. (1975), *Biochem. J. 149,* 155–167.

Pettigrew, G. W., and Schejter, A. (1974), *FEBS Lett. 43,* 131–134.

Salemme, R. R., Kraut, J., and Kamen, M. D. (1973), *J. Biol. Chem. 248,* 7701–7716.

Schejter, A., and George, P. (1964), *Biochemistry 3* 1045–1049.

Shechter, E., and Saludjian, P. (1967), *Biopolymers 5,* 788–790.

Takano, T., Kallai, O. B., Swanson, R., and Dickerson, R. E. (1973), *J. Biol. Chem. 248,* 5234–5255.

Weber, G., and Teale, W. J. (1959), *Discuss. Faraday Soc. No. 27,* 134–141.

Wilgus, H., and Stellwagen, E. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2892–2894.