Some electron-transfer reactions involving carbodi-imide-modified cytochrome *c*

Antony J. MATHEWS* and Thomas BRITTAIN

Department of Biochemistry, University of Auckland, Auckland, New Zealand

The reaction kinetics of native and carbodi-imide-modified tuna and horse heart cytochromes *c* with both a strong (dithionite) and a relatively weak (ascorbate) reducing agent were studied over a wide range of conditions. In their reactions with dithionite both the native and modified cytochromes exhibit single exponential time courses. The effects of dithionite concentration and ionic strength on the rate of the reduction are complex and can best be explained in terms of the model proposed by Lambeth & Palmer [(1973) *J. Biol. Chem.* **248**, 6095–6103]. According to this model, at low ionic strength the native proteins are reduced almost exclusively by $S_2O_4^{2-}$ whereas the modified proteins showed reactivity towards both $S_2O_4^{2-}$ and $SO_2^{\cdot-}$. These findings are interpreted in terms of the different charge characteristics of the carbodi-imide-modified proteins relative to the native proteins. The findings that the modified proteins react with ascorbate in a biphasic manner are explained as arising from ascorbate binding to a reducible form of the protein, before electron transfer, with an equilibrium between the ascorbate-reducible form of the protein and a non-reducible form. Estimates were obtained for both the ascorbate equilibrium binding constant and the rate constant for the internal electron transfer for both the native and modified horse and tuna proteins. The effect of pH on the reactions indicates that the active reductant in all cases is ascorbate $^{2-}$. The studies of ascorbate reactivity yield important information concerning the proposed correlation between ascorbate reducibility and the presence of a 695 nm-absorption band, and the study of dithionite reactivity illustrates the effect of protein charge and solution ionic strength on the relative contributions made by the species $SO_2^{\cdot-}$ and $S_2O_4^{2-}$ to the reduction of ferricytochrome *c*.

INTRODUCTION

Many investigators of electron transfer between cytochrome *c* and both small inorganic reductants and macromolecular species have suggested that the process of intramolecular electron exchange occurs via the exposed haem edge of cytochrome *c* [1–4]. Although many chemically modified species of cytochrome *c* have been prepared in attempts to probe the nature of the electron-transfer process [5], only the modification introduced by Timkovich [6] has successfully allowed modification of the exposed haem edge, via reaction of the haem propionate side chain with a water-soluble carbodi-imide. The derivative produced has a set of unusual properties, being ascorbate-reducible and yet lacking a 695 nm-absorption band [6]. As such, it represents an interesting system in which to investigate the correlation between Met-80 ligation and ascorbate-reducibility. A re-evaluation of EDC-modified cytochrome *c* has been reported recently [7].

The mechanism whereby ascorbate is able to reduce haem proteins is not well understood. It has been reported that at neutral pH cytochrome *c* reacts in a simple bimolecular fashion with ascorbate [8,9]. However, Pande & Myer [10] have presented evidence that reaction occurs via an initial formation of a

complex, possibly involving ascorbate binding at Arg-38. The binding process is followed by intramolecular electron transfer, which might occur, at least at lower temperature, by quantum tunnelling to the haem group [11]. The effect of pH on the reaction is marked, and it has been found that at alkaline pH the reaction occurs in two phases, one of which is associated with the conversion of the protein from a non-reducible into a reducible form [12]. A further investigation of the effect of pH has clearly shown that a significant effect on the reaction occurs as a consequence of the ionization of the ascorbate molecule, and this has allowed the identification of the double-deprotonated ascorbate dianion as the effective reducing agent [13]. The role that protein structure plays in these reactions has been studied. It has been shown from studies at high pH that ascorbate-reducibility correlates with the presence of a 695 nm-absorption band, which in turn correlates with Met-80 ligation to the haem iron atom [14]. It has also been shown that at neutral pH non-reducible forms may be produced by the presence of urea, and the transition leading to the formation of the non-reducible forms has been identified as that associated with loss of the 695 nm-absorption band [15,16]. From these studies it would thus appear that the presence of a 695 nm-absorption band is a prerequisite for ascorbate-

Abbreviation used: EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide ['1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide'].

* To whom correspondence should be sent, at present address: Department of Biochemistry, Rice University, Houston, TX 77251, U.S.A.

reducibility. However, a careful review of the literature dealing with the relationship between ascorbate-reducibility and the 695 nm-absorption band of ferricytochrome *c* shows several reports detailing the maintenance of ascorbate-reducibility in forms of cytochrome *c* that have greatly diminished absorption at 695 nm [17], or even complete loss of this band, as found for [methionine-80 sulphoxide]cytochrome *c* [18], [*N*-formyl-tryptophan-59]cytochrome *c* [19] and EDC-modified cytochrome *c* [6], the subject of the present study.

The mechanism of the dithionite reduction of cytochrome *c* has been studied by two groups [20,21]. Although the results in each case were very similar, different interpretations were made of the data. Creutz & Sutin [21] assumed that $S_2O_4^{2-}$ is the only active reducing agent, and as such were obliged to propose that a sulphite-ferrocycytochrome *c* species is formed in order to account for the complex concentration-dependence of the reaction. However, Lambeth & Palmer [20] considered the possibility that both $S_2O_4^{2-}$ and $SO_2^{\cdot-}$ might react with cytochrome *c*. It is known that these two species exist in equilibrium in solution [22], and on this basis Lambeth & Palmer [20] produced a more general and flexible model that can account for the dithionite reduction of many different metalloproteins. As such the Lambeth & Palmer [20] model has been used to analyse the data obtained in the present study.

MATERIALS AND METHODS

Materials

Albacore tuna were caught on long lines off the east coast of New Zealand's South Island, and the hearts were quickly removed and stored in solid CO_2 until used. Cytochrome *c* was obtained from tuna hearts by a modification of the method reported by Brautigan *et al.* [23]. In the present preparation centrifugation replaced filtration in the early steps of the previously reported procedure. The crude cytochrome was then recovered by batchwise adsorption on CM-cellulose rather than by adsorption on a column of Amberlite IRC-50. All other steps were as previously reported [23]. Horse heart cytochrome *c* type VI and EDC were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Deamidated and polymeric forms of the protein were removed by chromatography on CM-cellulose [23]. Chemical modification of both horse heart and tuna heart cytochromes *c* was performed exactly as previously described [6]. Monomeric protein was carefully separated from dimeric protein (resulting from EDC-promoted intermolecular cross-linking), by passage down a Sephadex G-50 column (83 cm $\times$ 3.2 cm diam.).

Stopped-flow experiments

Stopped-flow experiments were performed and analysed as previously described [24]. The ascorbate reduction reaction was followed at low reductant concentrations by monitoring the change in absorption as a function of time at 417 nm with an Aminco DW2a spectrophotometer (American Instrument Corp., Silver Spring, MD, USA). All dithionite solutions were standardized in order to determine the concentration of active reductant by titration against ferricyanide by previously reported procedures [21].

Analysis of dithionite reduction kinetics

Dithionite reduction kinetics were analysed according to the scheme proposed by Lambeth & Palmer [20]:

where $\text{Cyt}.c^{3+}$ and $\text{Cyt}.c^{2+}$ represent ferricytochrome *c* and ferrocycytochrome *c* respectively.

If $[S_2O_4^{2-}] > [\text{ferricytochrome}]$, $k_{+1} \gg k_{+2}$ and the dissociation equilibrium of $S_2O_4^{2-}$ is fast relative to k_{+4} , and both $SO_2^{\cdot-}$ and $S_2O_4^{2-}$ are considered as active reductants, the reduction of ferricytochrome is given by:

$$k_{\text{obs.}} = a[S_2O_4^{2-}]^{\frac{1}{2}} + b[S_2O_4^{2-}] \quad (1)$$

where $a = k_{+4}(k_{+3}/k_{-3})^{\frac{1}{2}}$ and $b = k_{+1}$.

In all the data reported in the present paper k_{+3}/k_{-3} has been corrected for the effect of ionic strength by using the data of Chien & Dickinson [22]. It should be noted that k_{+3}/k_{-3} has been found to be pH-independent [22]. According to eqn. (1), a plot of $k_{\text{obs.}}$ versus $[\text{dithionite}]^{\frac{1}{2}}$ should, if both $S_2O_4^{2-}$ and $SO_2^{\cdot-}$ are active reductants, yield an upward-curving plot in which the initial low-concentration data are dominated by the reactivity of $SO_2^{\cdot-}$. In the present study the values of a and b were determined by iterative fitting of the experimental data to eqn. (1) and minimizing the residual sum of the squares of the deviations.

RESULTS

Stopped-flow mixing of both the native and haem-modified proteins with excess dithionite produced reaction time courses consisting of a single-exponential process. At low ionic strength (0.09 M), plots of the observed rate of reduction versus the square root of the dithionite concentration yielded upward-curving plots (Fig. 1). Analysis of these plots according to the Lambeth & Palmer [20] model (eqn 1) yielded second-order rate constants for the reaction of $S_2O_4^{2-}$ and $SO_2^{\cdot-}$ with the various proteins (Table 1). The effect of changing ionic strength on the rate of dithionite reduction of modified horse and tuna heart cytochromes *c* is shown in Fig. 2.

At all ascorbate concentrations studied the reduction of the haem-modified proteins was found to be biphasic. The faster-phase rate constant was found to rate-limit at approx. 0.2 s^{-1} above 25 mM-ascorbate, whereas the slower process was found to be concentration-independent in rate at approx. 0.01 s^{-1} up to 100 mM-ascorbate (Fig. 3a). The kinetic difference spectra obtained for the two kinetic phases identified both processes as being associated with reduction of the modified cytochrome (Fig. 4). The proportion that each kinetic processes contributed to the total absorption change was found to be independent of the ascorbate concentration employed. At a fixed ascorbate concentration the rate of the faster phases was markedly pH-dependent, whereas the

Fig. 1. Dithionite-concentration-dependence of the rate of reduction of native and EDC-modified tuna and horse heart cytochromes *c*

The concentration-dependence of the rate of reduction of native (○) and EDC-modified (□) tuna heart cytochrome *c* is shown in (a) and that of native (○) and EDC-modified (□) horse heart cytochrome *c* in (b). In both cases the best-fit line is shown to eqn. (1) of the text. The best-fit parameters are shown in Table 1.

Table 1. Best-fit parameters for the second-order rate constants for the reduction of cytochromes *c* by $\text{S}_2\text{O}_4^{2-}$ and $\text{SO}_2^{\cdot-}$

k_{+1} and k_{+4} represent the rate constants for reduction by $\text{S}_2\text{O}_4^{2-}$ and $\text{SO}_2^{\cdot-}$ respectively (as determined by using the analysis procedure of Lambeth & Palmer [20]). Parameters were obtained by fitting experimental data to eqn. (1).

Cytochrome <i>c</i>	$10^{-5} \times k_{+1}$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	$10^{-7} \times k_{+4}$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)
Tuna heart, native	2.4	0.5
Tuna heart, EDC-modified	1.2	7.4
Horse heart, native	2	2
Horse heart, EDC-modified	3.5	18

slower-phase rate was pH-independent. The proportion that each process contributed to the overall absorption change was pH-independent. Measurement of the temperature-dependence of the two kinetic phases yielded activation energies of 33 kJ/mol and 20 kJ/mol for the faster and slower processes respectively.

Fig. 2. Effect of ionic strength on the rate of reduction of the EDC-modified tuna and horse heart cytochromes *c*

EDC-modified tuna (○) and horse heart (□) cytochromes *c* (4 μM) were reduced by 180 μM -dithionite at pH 7.0 in 0.1 M-Tris buffer supplemented with various amounts of NaCl.

DISCUSSION

A single kinetic phase was observed in all the cases where cytochrome *c* was rapidly mixed with dithionite. This indicated that for both the native and modified proteins a single kinetically active protein species is present in solution. By following the analytical procedures of Lambeth & Palmer [20], outlined previously, the dithionite-concentration-dependence of the pseudo-first-order rate constants for both the native and modified proteins were fitted to eqn. (1). The second-order rate constants derived for the reduction of native horse heart cytochrome *c* by $\text{SO}_2^{\cdot-}$ and $\text{S}_2\text{O}_4^{2-}$ as a function of ionic strength are shown in Table 2. It is clear from these data that the second-order rate constant for the reduction of native horse cytochrome *c* by $\text{S}_2\text{O}_4^{2-}$ (k_{+1}) decreases with increasing ionic strength, whereas the rate for reduction by $\text{SO}_2^{\cdot-}$ (k_{+4}) increases. These findings suggest that the apparent lack of reactivity of native horse heart cytochrome *c* with $\text{SO}_2^{\cdot-}$ arises from an ionic-strength effect. From the ionic-strength-dependence of k_{+1} and k_{+4} it appears that $\text{S}_2\text{O}_4^{2-}$ interacts with the protein at a positively charged centre, possibly the periphery of the haem crevice, whereas $\text{SO}_2^{\cdot-}$ appears to interact with a negatively charged centre [25]. It is tempting to postulate that this negative centre is the carboxylate group of the less-buried haem propionate group. These data are in accord with the effect of ionic strength on the reduction of haem nonapeptide with dithionite, which has been interpreted as arising from attack of $\text{SO}_2^{\cdot-}$ at a negatively charged site [26]. The data of Table 1 indicate that the modification of both horse and tuna cytochromes *c* with EDC drastically affects the protein reactivity towards $\text{SO}_2^{\cdot-}$ while little affecting the reaction with $\text{S}_2\text{O}_4^{2-}$. In terms of the charge character of the reaction site, the change in reactivity towards $\text{SO}_2^{\cdot-}$ could well arise from the loss of negative charge on a carboxylate group (possibly the haem propionate group) as a result of chemical modification. However, in the absence of direct evidence for this proposal we cannot rule out the possibility that increased reactivity with $\text{SO}_2^{\cdot-}$ merely reflects the presence of a more-open,

Fig. 3. Ascorbate-concentration-dependence of the rate of reduction of native and EDC-modified tuna and horse heart cytochromes *c*

The concentration-dependence of the fast phase (● and ■) and slow phase (○ and □) observed in the ascorbate reduction of EDC-modified cytochromes *c* is shown in (a). Data for both the tuna (○ and ●) and horse (□ and ■) proteins are shown. In (b) double-reciprocal plots of the rate data for the fast phase of reduction of the modified tuna (■) and horse (□) proteins are shown together with those for the native tuna (●) and native horse (○) proteins.

disrupted, haem crevice in the chemically modified species.

Reaction of the EDC-modified proteins with ascorbate produces biphasic reaction traces. The rate-limiting faster phase can be accounted for by the mechanism proposed by Myer *et al.* [16], which involves complex-formation before electron transfer. A pseudo-first-order rate limit for the reduction of cytochrome *c* at high concentrations of 4-acetylcatechol and hydrocaffeic acid has been observed by Saleem & Wilson [27]. These reducing agents act by a mechanism analogous to that of ascorbate [27]. The slower phase, the rate of which is independent of ascorbate concentration, exhibits a kinetic difference spectrum that is indistinguishable from

Fig. 4. Difference spectra for the ascorbate reduction of EDC-modified tuna and horse heart cytochromes *c*

Tuna (a) and horse heart (b) cytochromes *c* (4 μ M) were reduced with 100 mM-ascorbate at pH 7.0 in 0.1 M-Tris buffer. A comparison is shown of the static (○) difference spectrum and that of the fast (●) and the slow (□) kinetic phases for each protein.

Table 2. Ionic-strength-dependence of the rates of reduction of native horse heart cytochrome *c* by $\text{SO}_2^{\cdot-}$ and $\text{S}_2\text{O}_4^{2-}$

k_{+1} and k_{+4} represent the rates of reduction by $\text{S}_2\text{O}_4^{2-}$ and $\text{SO}_2^{\cdot-}$ respectively, as determined by using the analysis procedure of Lambeth & Palmer [20].

<i>I</i>	$10^{-5} \times k_{+1}$ (M ⁻¹ ·s ⁻¹)	$10^{-7} \times k_{+4}$ (M ⁻¹ ·s ⁻¹)
0.09	2	2
0.30	15*	4.1*
0.94	0.08†	8†

* Data from Lambeth & Palmer [20].

† Data from Creutz & Sutin [21].

that of the reduction process. It therefore appears that the slow phase is spectrally silent and is being detected by its effect on a subsequent relatively rapid, reduction process. The pH-independence of the slow-phase rate further indicates that this reaction is not a reduction, but a protein conformation change. Furthermore, as the relative contributions of the two kinetic phases to the total absorption change are constant, it would appear that the reducible and non-reducible forms of the protein are only in slow equilibrium. A kinetic model consistent with the presence and character of two kinetic phases as outlined above is that proposed by Myer *et al.* [16] to account for the reduction of native cytochrome *c* in the presence of urea, namely:

where $k_1 \ll k_3$ and $\text{Cyt.}c^*$ and $\text{Cyt.}c$ represent non-reducible and reducible forms of the protein respectively. The pH-dependence of the rate of the faster phase at constant ascorbate concentration is indicative of ascorbate^{2-} being the active reductant [13]. In this case a value for k_{+1}/k_{-1} (K_u) can be obtained from the relative proportions that the fast and the slow kinetic phases contribute to the overall absorption change (0.35 for the modified tuna protein and 0.5 for the modified horse heart protein). Myer *et al.* [16] have shown that for this scheme:

$$1/k_f = 1/k_{+3} + (1 + K_u)/K_{ss}k_{+3}[\text{ascorbate}]$$

where $K_u = k_{+1}/k_{-1}$ and K_{ss} is the equilibrium constant governing the complex-formation between ascorbate and the protein. Plots of $1/k_f$ versus $1/[\text{ascorbate}]$ should thus yield straight lines (Fig. 3b). For these plots it can be shown that:

$$K_{ss} = (1 + K_u)/(\text{slope/intercept})$$

The resultant values of k_3 and K_{ss} are shown in Table 3. For native horse heart cytochrome *c* the value of K_{ss} increases markedly at low ionic strength whereas the value of k_{+3} , the intramolecular electron-transfer rate, decreases slightly. The significant change in K_{ss} with ionic strength is in accord with the suggestion that the ascorbate^{2-} ion interacts with Arg-38 in the horse heart protein [10].

The significant change in K_{ss} resultant on modification of the protein could well arise from the removal of a negative charge close to the binding site brought about by conversion of a carboxylate group into an amide group by reaction with EDC. The lowering of the value of the rate of the intramolecular electron-transfer rate produced by modification could arise from slight alteration in the structure of the electron-transfer path,

particularly since evidence has been obtained that this process involves quantum tunnelling [11].

It is noteworthy that the rate of the slower phase (k_{+1}) obtained in these studies has a value of approx. 0.02 s^{-1} whereas the rate of the alkaline isomerization, which gives a low rate for the reduction of native cytochrome *c* at alkaline pH, has a value of 0.04 s^{-1} [8]. Similar rates ($0.05 \pm 0.025 \text{ s}^{-1}$) have also been obtained for the slow phase observed in the reduction of native cytochrome *c* at neutral pH in the presence of urea and also in the case of the unfolding and refolding of urea-denatured cytochrome *c* [16,28]. A similar structural alteration may therefore be involved in each case. In the case of the effect

of urea on native cytochrome *c* the induced transition leading to loss of ascorbate-reducibility has been correlated with the loss of the 695 nm-absorption band [17].

In the present study neither of the EDC-modified cytochromes possesses a 695 nm-absorption band, yet ascorbate-reducibility is retained, as has been observed for other chemically modified cytochrome *c* species [18, 19]. Clearly, the widely held view that the presence of a 695 nm-absorption band, or, equivalently, normal Met-80-iron ligation, is necessary for the maintenance of ascorbate-reducibility is incorrect. Thus it remains to reconcile the findings presented in the present study with the widespread observation of species of ferricytochrome *c* lacking both a 695 nm-absorption band and ascorbate-reducibility [8,12].

It is clear that the EDC-modified proteins lack native-protein-like Met-80 co-ordination of the haem iron. However, it is possible that this ligand still remains weakly co-ordinated to the iron atom in a fraction of molecules present at equilibrium (i.e. that fraction which undergoes reduction in the fast phase) to a degree that allows electron transfer from ascorbate to the haem iron (although at lower efficiency than with the native protein) and yet shows no evidence of a 695 nm-absorption band. Support for residual Met-80 co-ordination of the haem iron in the absence of a 695 nm-absorption band comes from several sources. Eaton & Hochstrasser [29] have proposed that the weak 695 nm-absorption band is due to a porphyrin $a_{2u}(\pi) \rightarrow \text{iron } a_{1g}(d_z^2)$ charge-transfer transition, with the energy of the transition being extremely sensitive to the placement of the Met-80 sulphur ligand relative to the haem iron. It has been shown by Myer *et al.* [17] that, of the total absorption change at 695 nm due to urea denaturation of ferricytochrome *c*, some two-thirds is lost before actual disruption of the Met-80 sulphur-haem iron bond. The initial decrease in absorption was assigned to protein conformation changes. The sensitivity of the 695 nm-absorption band to the position of the Met-80 residue has also been demonstrated by Senn *et al.* [14,30], who showed that the chirality of the axially bound Met-80 residue determined the distribution of electron density in the haem group, and the sign of the 695 nm c.d. band of cytochrome *c*. These findings suggest that subtle alteration in the position of the Met-80 residue can markedly alter the intensity of the 695 nm-absorption band.

Table 3. Effect of EDC modification and ionic strength on the rate of reaction of cytochromes *c* with ascorbate

Cytochrome <i>c</i>	$k_{+3} \text{ (s}^{-1}\text{)}$	$K_{ss} \text{ (M}^{-1}\text{)}$	<i>I</i>
Tuna heart, EDC-modified	0.18	240	0.09
Tuna heart, native	0.92	13	0.09
Horse heart, EDC-modified	0.33	203	0.09
Horse heart, native	0.77	23	0.09
	3.0*	5.9*	0.75*

* From the data of Myer *et al.* [16].

The extent of Met-80 co-ordination of the haem iron may confer ascorbate-reducibility by virtue of the central role of this ligand in maintaining the redox potential of cytochrome *c*. Pande & Myer [31] have shown that the redox potential of cytochrome *c* is relatively insensitive to urea-induced protein conformation changes, and that drastic diminution of the redox potential only occurred as Met-80 ligation to the haem iron was lost. In the case of non-ascorbate-reducible species, such as the fraction of EDC-modified cytochrome reduced in the slow phase or carboxymethylated cytochrome *c* [16,32], the methionine ligand may be completely lost from the co-ordination sphere of the haem iron, yielding a five-co-ordinate species. This in turn could lower the redox potentials of the species to values that preclude reduction by ascorbate (E'_0 (ascorbate) = +0.058 V, at pH 7.0 and 30 °C [33]). The reduction potentials of the ascorbate-reducible EDC-modified cytochrome *c* [6], [methionine-80 sulphoxide]cytochrome *c* [18] and [*N*-formyltryptophan-59]cytochrome *c* [19] have been reported as +0.105 V, +0.184 V and +0.085 V respectively. In the case of reduction by $\text{SO}_2^{\cdot-}$ and $\text{S}_2\text{O}_4^{2-}$ (E'_0 = -0.66 V and -0.386 V respectively at pH 7.0 and 25 °C [34]), the redox potentials of the reductants are such that reduction of all protein structures is possible.

REFERENCES

- Margoliash, E. & Bosshard, H. R. (1983) *Trends Biochem. Sci.* **8**, 316–320
- Marcus, R. A. & Sutin, N. (1985) *Biochim. Biophys. Acta* **811**, 265–322
- Hodges, H. L., Holwerda, R. A. & Gray, H. B. (1974) *J. Am. Chem. Soc.* **96**, 3132–3137
- McArdle, J. V., Gray, H. B., Creutz, C. & Sutin, N. (1974) *J. Am. Chem. Soc.* **96**, 5737–5741
- Ferguson-Miller, S., Brautigan, D. C. & Margoliash, E. (1979) in *The Porphyrins* (Dolphin, D., ed.), vol. 7, pp. 150–240, Academic Press, London.
- Timkovich, R. (1980) *Biochem. J.* **185**, 47–57
- Mathews, A. J. & Brittain, T. (1986) *Biochem. J.* **240**, 181–187
- Wilson, M. T. & Greenwood, C. (1971) *Eur. J. Biochem.* **22**, 11–18
- Williams, N. H. & Yandell, J. K. (1985) *Biochim. Biophys. Acta* **810**, 274–277
- Pande, J. & Myer, Y. P. (1980) *J. Biol. Chem.* **255**, 11094–11097
- Myer, Y. P. & Kumar, S. (1984) *J. Biol. Chem.* **259**, 8144–8150
- Greenwood, C. & Palmer, G. (1965) *J. Biol. Chem.* **240**, 3660–3663
- Al-Ayash, A. I. & Wilson, M. T. (1979) *Biochem. J.* **177**, 641–648
- Senn, H., Keller, R. M. & Wüthrich, K. (1980) *Biochem. Biophys. Res. Commun.* **92**, 1362–1369
- Schejter, A., Glauser, S. C., George, P. & Margolish, E. (1963) *Biochim. Biophys. Acta* **73**, 641–643
- Myer, Y. P., Thallam, K. K. & Pande, A. (1980) *J. Biol. Chem.* **255**, 9666–9673
- Myer, Y. P., MacDonald, L. H., Verma, B. C. & Pande, A. (1980) *Biochemistry* **19**, 199–207
- Ivanetich, K. M., Bradshaw, J. J. & Kaminsky, L. S. (1976) *Biochemistry* **15**, 1144–1153
- Ercińska, M. (1975) *Arch. Biochem. Biophys.* **169**, 199–208
- Lambeth, D. O. & Palmer, G. (1973) *J. Biol. Chem.* **248**, 6095–6103
- Creutz, C. & Sutin, N. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1701–1703
- Chien, J. C. W. & Dickinson, L. C. (1978) *J. Biol. Chem.* **253**, 6965–6972
- Brautigan, D. L., Ferguson-Miller, S. & Margoliash, E. (1978) *Methods Enzymol.* **53**, 128–164
- Brittain, T. (1980) *Biochem. J.* **187**, 803–804
- Ilan, Y., Shafferman, A., Feinberg, B. A. & Lau, Y. K. (1979) *Biochim. Biophys. Acta* **548**, 565–578
- Kazmi, S. A., Mills, M. A., Pitluk, Z. W. & Scott, R. A. (1985) *J. Inorg. Biochem.* **24**, 9–12
- Saleem, M. M. M. & Wilson, M. T. (1982) *Biochem. J.* **201**, 433–444
- Myer, Y. P., Pande, A. & Saturno, A. F. (1981) *J. Biol. Chem.* **256**, 1576–1581
- Eaton, W. A. & Hochstrasser, R. M. (1967) *J. Chem. Phys.* **46**, 2533–2539
- Senn, H. & Wüthrich, K. (1985) *Q. Rev. Biophys.* **18**, 111–134
- Pande, A. & Myer, Y. P. (1978) *Biochem. Biophys. Res. Commun.* **85**, 7–13
- Wilson, M. T., Brunori, M., Rotilio, G. C. & Antonini, E. (1973) *J. Biol. Chem.* **248**, 8162–8169
- Ball, E. G. (1937) *J. Biol. Chem.* **118**, 219–239
- Mayhew, S. G. (1978) *Eur. J. Biochem.* **85**, 535–547

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