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Characterisation of a near infra-red absorption band of the *Escherichia coli* quinol oxidase, cytochrome *o*, which is attributable to the high-spin ferrous haem of the binuclear site

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The bacterial quinol oxidase, cytochrome *o*, is an enzyme which is highly analogous to the better known cytochrome *c* oxidase, cytochrome aa_3, but with the important difference that it lacks the near infra-red absorbing pigment Cu_A. In this article we report an absorption band in the near IR spectrum of cytochrome *o* with a maximal absorption at 758 nm, and which is attributable to the ferrous high-spin haem. The 758 nm band has an extinction coefficient of 0.2–0.3 $mM^{-1} \cdot cm^{-1}$ at 758–800 nm. This region in cytochrome aa_3 is dominated by the Cu_A absorption. The 758 nm absorption is lost on addition of CO or cyanide to the reduced enzyme. The carbon monoxide compound of cytochrome *o* also has absorbance bands in the near infra-red, and these may be attributable to a low-spin ferrous haem compound.

Cytochrome *o*; Quinol oxidase; Oxygen reduction; Respiratory chain; Oxidative phosphorylation; *Escherichia coli*

1. INTRODUCTION

Cytochrome *o* is a membrane-bound terminal-respiratory oxidase, one of two which *Escherichia coli* can synthesize. The other, expressed predominantly under conditions of low oxygen tension, is cytochrome *bd*. Cytochrome *o* is comprised of four subunits and contains a low-spin and a high-spin iron-haem and a copper centre (Cu_B) as prosthetic groups (all located in subunit I). The latter two form a binuclear (Fe–Cu) site which is the catalytic core of the oxygen reduction reaction. Although widely spread and probably the most dominant respiratory O_2 reductase in nature, cytochrome *o* is much less studied than the cytochrome *c* oxidase, cytochrome aa_3 [1,2]. Nonetheless, the two enzymes are closely related which makes the cytochrome *o* a system worthy of study for reasons beyond its own intrinsic interest. Cytochrome *o* is similar to cytochrome aa_3 in terms of: (i) sequence homology of subunit I [3] (ii) some sequence homology/similarity in subunit II but lacking the putative Cu_A binding region of cytochrome aa_3 [3], (iii) the presence of a copper-haem interaction forming the binuclear O_2 reducing site [4,5], (iv) haem–haem interactions, between the high- and low-spin haems which result in spectral and redox effects [4,5], (v) the alignment of the haems with respect to the cytoplasmic membrane [6,7], (vi) vectorial transmembrane proton pumping during turnover [8], and (vii) reactions of the binuclear centre with exogenous ligands [9,10]. The two enzymes differ in the lack of the Cu_A site in cytochrome *o*, a deficiency established by metal analysis, preliminary EXAFS studies [11], optical spectra [11], electron paramagnetic resonance studies [5,6] and sequence considerations [3]. This difference is important because the near IR spectrum of cytochrome *o* is free from the large broad absorption of Cu_A which dominates in cytochrome aa_3 and it is in this region in which we report the existence and preliminary characterization of an absorption band attributable to ferrous high-spin haem. The reason for the lack of Cu_A in cytochrome *o* most logically stems from it being a quinol oxidase as opposed to a cytochrome *c* oxidase, and thus not requiring a more hydrophilic electron pathway. A more visibly striking but probably minor functional difference between the two enzymes is the colour of the isolated enzyme, red instead of green. This results from the different haem types. Cytochrome *o* contains protoporphyrin IX derived haems, at least one of which has a hydrophobic 17-carbon hydroxyethylfarnesyl side chain, making it structurally very similar to the A-type haems of cytochrome aa_3 but differing in having a methyl substituent on the porphyrin ring instead of formyl [9].

The near IR spectra of haemoproteins contain much spectral information, albeit with low extinction [12]. There have been several experimental and theoretical analyses of haem near IR spectra [13]. Published work on haemoglobin and myoglobin near IR spectra can serve as a preliminary reference for the interpretation of

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the near IR spectra of cytochrome *o*. Near IR absorption bands similar to that reported herein, around 760 nm, have been extensively studied in myoglobin and haemoglobin. The absorption is well characterized and is assigned to a charge transfer between the porphyrin and the iron [12,14]. This band has been denoted band III and is expected to be very sensitive to the ligand field at the iron. Iizuka et al. [15] called this band the 'conformation band' because of its sensitivity to structure at the haem site. For haemoglobins and oxidases the ligand field parameters are connected to the haem pocket geometry via the Fe-proximal histidine bond. Thus this absorption can provide in oxidases, (as it has in haemoglobins) a sensitive indicator of alterations in the haem pocket structure.

2. MATERIALS AND METHODS

Cytochrome *o* was isolated and purified from *E. coli* RG145, a strain with amplified expression of cytochrome *o* and unable to express the other quinol oxidase, cytochrome *bd* [16]. Cells were grown and harvested, and membrane fragments were prepared, as previously described [5]. The cytochrome was solubilised by octyl-glucoside and purified according to a protocol developed in the laboratory of Prof. R.B. Gennis (personal communication), based on the method of Matsushita et al. [17].

Optically monitored redox potentiometry was carried out in a 1-cm pathlength cuvette with a glassy carbon measuring electrode and a Ag/AgCl reference [18,19]. The redox mediators were 50 μM each of hexammineruthenium chloride, 5-ethyl phenazinium ethosulphate, 5-methyl phenazinium methosulphate, trimethyl-1,4-benzoquinone, diaminodurene, and 1,4-naphthoquinone-2,5-disulphonate. The medium was 25 mM potassium phosphate, 1 mM EDTA, and 5 mM TRIS-HCl at pH 7.0, with 0.05% lauryl sarkosine and 30 units·ml^{-1} catalase. Solutions of sodium dithionite and potassium ferricyanide were used as titrants.

Infra-red spectroscopy was performed with a single beam spectrophotometer built in-house, incorporating a ruled grating (1 micron blaze; 600 lines/mm) and a photodiode detector (1 cm^2 active area) which was protected by appropriate optical filters.

The same machinery was used for room-temperature flash photolysis of the carbon monoxide adduct. The flash was provided by a xenon flashlamp (E.G. & G. FXP852) connected to a 15 μF, capacitor charged to 1000 V, i.e. 7.5 J/flash. The flash had a duration of 2 μs at half maximal intensity. Two fibre-optic bundles (1 cm diameter) placed about 2.5 cm from the arc (length 3 mm) were used to collect the light and guide it, perpendicular to the measuring beam, to opposite sides of a 10-mm measuring beam pathlength cuvette. The optical pathlength of the actinic light was 1 cm or 0.3 cm, dependent on sample size. In order to minimise flash artefacts, BG 3 and BG 39 filters were placed between the lamp and the fibre-optic bundles and in this case it was approximately 5% saturating for carbon monoxide photodissociation. The photomultiplier was protected with complementary filters (WG360, GG530, RG610, RG715 or OX5495) as appropriate.

3. RESULTS

Difference spectra of reduced forms of cytochrome *o* vs. oxidized enzyme are shown in Fig. 1a. Spectrum A is a [dithionite reduced] *minus* [oxidised] difference spectrum. This spectrum shows an α-band peak in the 560 nm region attributable to the ferrous low-spin haem. The trough in the 640 nm region is due to a band of the ferric high-spin haem (of the binuclear site) which is lost on reduction. In the near IR a feature is shown, inset, at enhanced gain with a peak at 758 nm ($\varepsilon \sim 0.25$ mM^{-1}·cm^{-1}, vs. 800 nm in the difference spectrum).

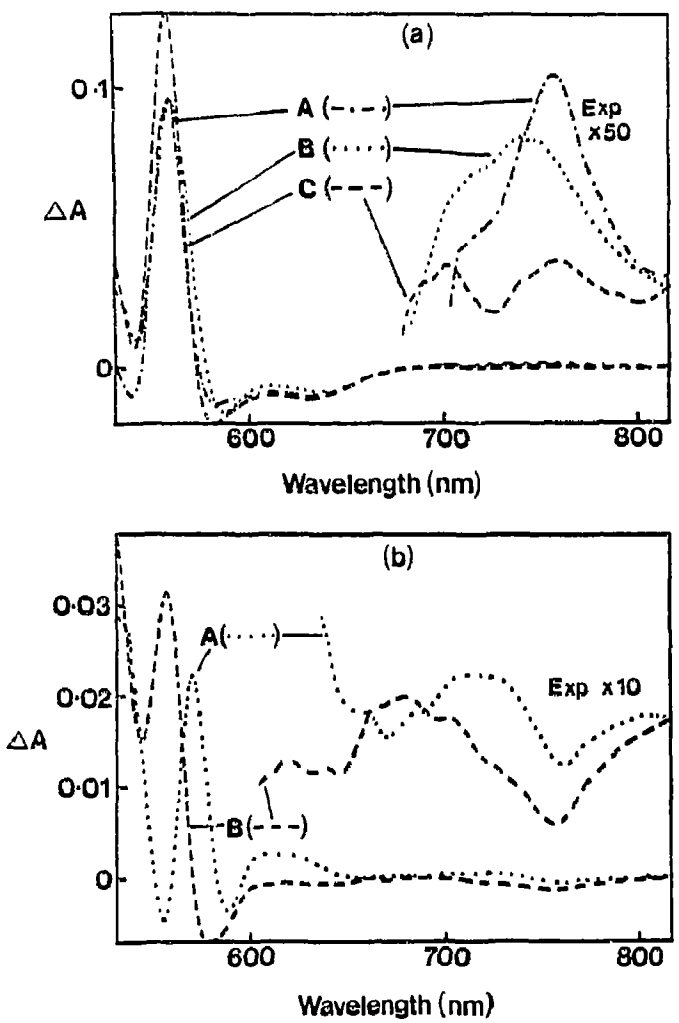

Fig. 1. Spectra of cytochrome *o* in the visible and near infra-red. (a) Reduced minus oxidised difference spectra: A. [Reduced *minus* oxidised] (-•-•-•); B. [Reduced *plus* CO] *minus* [oxidised] (••••••); C. [Reduced *plus* cyanide] *minus* [oxidised] (---). (b) [Reduced *plus* ligand] *minus* [reduced] difference spectra; A. [Reduced *plus* CO] *minus* reduced] (••••••); B. [Reduced *plus* cyanide] *minus* [reduced] (---). The cuvette contained 5.5 μM cytochrome *o* in 0.03% lauryl sarcosine, 33 mM potassium phosphate, 3.3 mM Tris-HCl and 1.3 mM EDTA, pH 7.0. The sample was reduced with a grain of dithionite. The CO-compound was formed by gently stirring the sample under a CO atmosphere for at least 5 min. The cyanide compound was formed by the addition of a pH-adjusted stock solution of potassium cyanide, to a final concentration of 166 mM. Spectra were recorded at room temperature and each spectrum is the average of 10 scans.

The addition of carbon monoxide or cyanide to the reduced enzyme causes spectral changes in both the α-band and near IR regions (spectra B and C in Fig. 1a). The addition of CO causes a loss of the 758 nm absorption and the appearance of a smaller absorbance with a peak in the region of 745 nm. The extinction coefficient of the CO-compound at this wavelength is approx.

0.05 $mM^{-1} \cdot cm^{-1}$ at 750–700 nm. This CO-induced spectrum is also apparent in the [reduced *plus* CO] *minus* [reduced] difference spectrum shown in Fig. 1b, trace A, where its presence causes the appearance of a trough and peak. The effect of cyanide is to bleach the 758 nm band (Fig. 1a, trace C) and this is also clearly seen in the [reduced *plus* cyanide] *minus* [reduced] difference spectrum (Fig. 1b, trace B). Some small unexplained features at 700–750 nm remain in the [reduced *plus* cyanide] *minus* [oxidised] spectrum.

Fig. 2 shows a flash-photolysis spectrum of the CO-compound of cytochrome *o* taken 5.5 ms after the flash, and at room temperature. The inset shows the near IR absorption changes at a 10-fold gain. The spectrum is the inverse of the CO-difference spectrum but the methodology gives it a more reliable baseline. The flash-photolysis spectrum confirms all the features of the CO-difference spectrum, including the broad band in the 640 nm region and the peak at around 760 nm. The apparent peak position of the near IR band is 758 nm in the [reduced] *minus* [oxidised] spectrum (Fig. 1a, trace A), but at slightly longer wavelength in the CO-difference (Fig. 1b, trace B) and CO-photolysis spectra (Fig. 2). This is probably due to the underlying (negative) spectrum of the CO-compound in the latter cases.

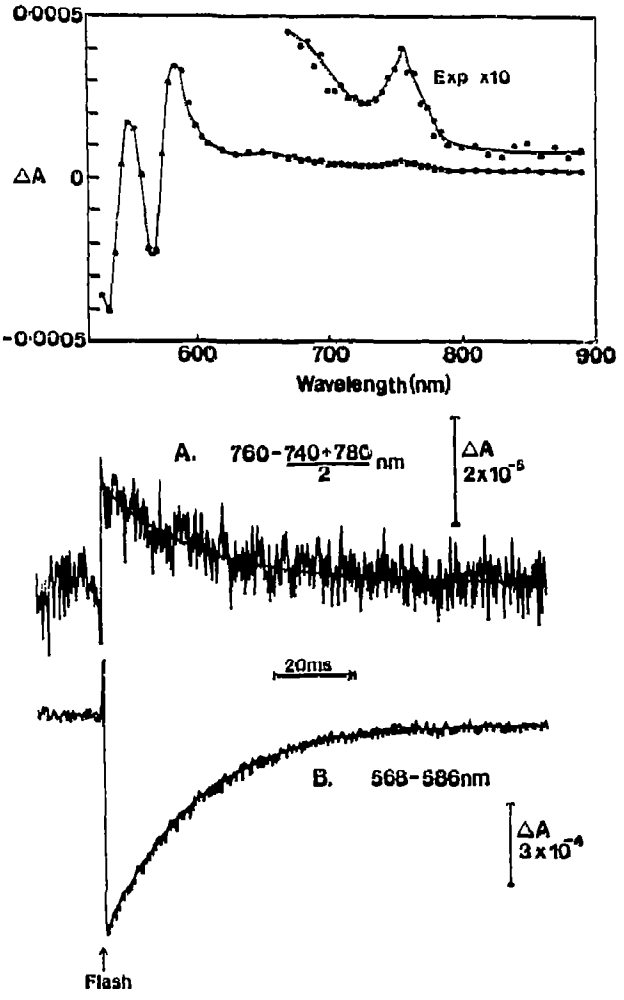

Fig. 2. Room temperature flash photolysis of the CO compound. Top: CO-photolysis spectrum, 5.5 ms after the flash. Bottom: transient kinetics of room temperature recombination of CO measured in (A) the near IR band at (760–(740+780)/2) nm (50 averages per wavelength); (B) the visible region at (568–586) nm (10 averages per wavelength). The photolysis spectrum was assembled in sections (540–615 nm, 610–715 nm, 700–790 nm, and 750–820 nm) and each point is the average of either 10 (visible region) or 50 (near IR region) flashes at that wavelength. The kinetic transients are both fitted to a first order relaxation with a rate constant of 46 s^{-1}. The cuvette contained 2.2 μM cytochrome *o* in 50 mM potassium phosphate, 2 mM EDTA, 0.1% lauryl-maltoside and 0.013% lauryl sarcosine at pH 7 and was reduced with a grain of dithionite. The buffer was saturated with CO prior to addition of enzyme and the space above the mixture was a CO atmosphere.

Progress curves of CO-recombination at room temperature, as monitored by the visible (568–586 nm) and near IR (760−0.5 × (740 + 780) nm) features, are shown in Fig. 2B. In both cases the relaxations are well fitted as first order processes with rate constants of 46 s^{-1}, further indicating that the absorption changes arise from the same process. These kinetic parameters are the same for all features of the CO-photolysis spectrum.

There have been a number of potentiometric studies on cytochrome *o*, but none have extended the observations to the near IR region [1,4,20,21]. To check that the redox behaviour of the 758 nm band corresponds to that expected of the high-spin ferrous haem we conducted redox titrations of the 758 nm peak and also monitored the absorbance changes at 640 nm and 564 nm. The 640 nm change is attributed to high-spin ferric haem and the 564 nm band to low-spin ferrous haem [21]. Spectra poised at different redox potentials, taken against an oxidised baseline, are shown in Fig. 3. The

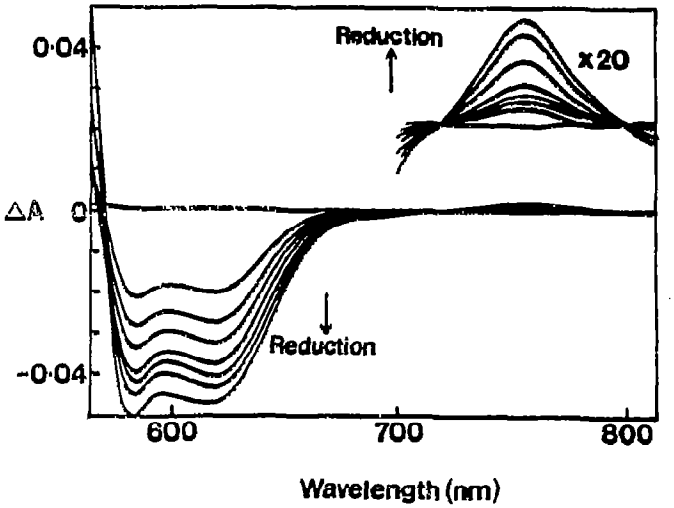

Fig. 3. Spectra obtained during a redox titration of cytochrome *o*. Redox titrations were conducted under a nitrogen atmosphere at room temperature. The reaction mixture is given in section 2. Cytochrome *o* was present at 8 μM and the baseline was recorded at E_h = +275 mV. The spectra are in the E_h sequence: 275, 215, 200, 185, 160, 110, 0 and −115 mV from the horizontal centre line. Each spectrum is an average of 10 scans.

potentiometric data indicate two redox transitions in all bands, at approx. 255 mV and 110 mV. The ratio of the absorption changes in each transition differs at each wavelength monitored. This is due to the anticooperativity described in this system [4] and is further complicated by haem–haem interactions leading to spectral shifts and apparent extinction coefficient changes for transitions between different states [4,5,21].

4. DISCUSSION

The results show that the 758 nm band appears in the reduced form of the enzyme in the absence of exogenous ligands. The absorption is lost on ligation of CO or cyanide, both of which are ligands to high-spin ferrous haems and which bind in analogous systems to form low-spin ligand complexes. The 758 nm band titrates potentiometrically (not shown) with similar midpoint potentials to the other haem features in the enzyme. The biphasic titration behaviour of all haem spectrum features arises from haem–haem interactions both in cytochrome *o* [4] and in cytochrome *c* oxidase [22]. The titration of the 758 nm band isopotentially with the other haem features (apparent E_{m7} values of approx. 255 and 110 mV) further confirms the association of this band with haem and eliminates Cu_B (E_{m7} of approx. 320 mV [4]) as a source of the signal.

The wavelength maximum in the reduced *minus* oxidized spectrum (Fig. 1aA) is at 758 nm and has a half peak width of approx. 30 nm. The peak position is very close to those of the analogous features in deoxyMb and deoxyHb (761 and 759 nm at 298 K, [14,23,24], which also have linewidths of approx. 30 nm at room temperature. The band in cytochrome *o* has an ε of approx. 0.25 $mM^{-1}\cdot cm^{-1}$ in the difference spectrum (758 minus 800 nm), which is also similar to deoxyMb and deoxyHb. The ∽760 nm band has been assigned as a charge transfer transition between the porphyrin π system and the 'iron π system' ($a_{2u}(\pi)\rightarrow d_{zy}$) [12,14] or to a $d_{x^2-y^2}\rightarrow e_g(\pi^*)$ transition [25]. Herein it is sufficient to describe it as a charge-transfer transition.

The addition of CO and cyanide to deoxyMb or deoxyHb causes the compound to go low-spin with consequent loss of the 760 nm band. Cyanide and CO ligation to ferrous cytochrome aa_3 also causes the haem a_3 to go low-spin [26], and we suggest that this is also the case in cytochrome *o*. However, the detection of an absorbance at approx. 745 nm attributable to the CO-compound (ε_{mM} approx. 0.05 $mM^{-1}\cdot cm^{-1}$ at 745–700 nm) is interesting and requires further investigation. Horse carboxyHb has bands at 630 nm (visible in cytochrome *o* as a trough in Fig. 1bA), 720 nm and 800 nm which have been attributed to (*d–d*) transition of the low-spin compound [27]. The absorption of the cytochrome *o*–CO compound in the 745 nm region is possibly the equivalent to the carboxyHb 720 nm band.

The near IR region of haemoproteins contains a number of structurally and chemically sensitive transitions. The detection of such features in cytochrome *o* should provide a new sensitive probe for the binuclear centre and its environment.

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REFERENCES

[1] Poole, R.K. and Ingledew, W.J., in: *Escherichia coli* and *Salmonella typhimurium* Cellular and Molecular Biology, (F.C. Neidhardt, Ed.), Pub. Amer. Soc. Microbiol. 1987, pp. 170–200.
[2] Poole, R.K. (1983) Biochim. Biophys. Acta 726, 205–243.
[3] Chepuri, V., Lemieux, L., Au, D.C.-T. and Gennis, R.B. (1990) J. Biol. Chem. 265, 11185–11192.
[4] Salerno, J.C., Bolgiano, B., Poole, R.K., Gennis, R.B. and Ingledew, W.J. (1990) J. Biol. Chem. 265, 4364–4368.
[5] Salerno, J.C., Bolgiano, B. and Ingledew, W.J. (1989) FEBS Lett. 247, 101–105.
[6] Salerno, J.C. and Ingledew, W.J. (1991) Eur. J. Biochem. 198, 789–792.
[7] Blum, H., Harmon, H.J., Leigh, J.S. and Chance, B. (1978) Biochim. Biophys. Acta 502, 1–10.
[8] Puustinen, A., Finel, M., Virkki, M. and Wikstrom, M. (1989) FEBS Lett. 249, 163–167.
[9] Puustinen, A. and Wikstrom, M. (1991) Proc. Natl. Acad. Sci. 88, 6122–6126.
[10] Moody, A.J., Cooper, C.E. and Rich, P.R. (1991) Biochim. Biophys. Acta 1059, 189–207.
[11] Ingledew, W.J. and Bacon, M. (1991) Biochem. Soc. Trans. 19, 1298–1300.
[12] Eaton, W.A., Hanson, L.K., Stephens, P.J., Sutherland, J.C. and Dunn, J.B.R. (1978) J. Am. Chem. Soc. 100, 4991–5003.
[13] Gouterman, M., in: The Porphyrins, Vol. III, (D. Dolphin, Ed.), Academic Press, N.Y., 1978, pp. 1–165.
[14] Chavez, M.D., Courtney, S.H., Chance, M.R., Kiula, D., Nocek, J., Hoffman, B.M., Friedman, J.M. and Ondrias, M.R. (1990) Biochemistry 29, 4844–4852.
[15] Iizuka, T., Yamamota, H. and Yonetani, T. (1974) Biochim. Biophys. Acta 371, 126–139.
[16] Au, D.C.-T. and Gennis, R.B. (1987) J. Bacteriol. 169, 3237–3242.
[17] Matsushita, K., Patel, L. and Kaback, H.R. (1986) Methods Enzymol. 126, 113–122.
[18] Dutton, P.L (1978) Methods Enzymol. 54, 411–435.
[19] Rich, P.R., Jeal, A.E., Madgwick, S.A. and Moody, A.J. (1990) Biochim. Biophys. Acta 1018, 29–40.
[20] Withers, H.K. and Bragg, P.D. (1990) Biochem. Cell Biol. 68, 83–90.
[21] Bolgiano, B., Salmon, I., Ingledew, W.J. and Poole, R.K. (1991) Biochem. J. 274, 723–730.
[22] Wilson, D.F., Lindsay, J.G. and Brocklehurst, E.S. (1972) Biochim. Biophys. Acta 256, 277–286.
[23] Adar, F. (1978) in: The Porphyrins, Vol III, (D. Dolphin, Ed.), Academic Press, N.Y., 1978, pp. 167–210.
[24] Fiamingo, F.G. and Alben, J.O. (1985) Biochemistry 24, 7964–7970.
[25] Makinen, M.W. and Churg, A.K., in: Iron Porphyrins, (A.B.P. Lever and H.B. Gray, Eds.) Part I, Chapt. 3, Addison-Wesley, Reading, MA, 1983, pp. 141–235.
[26] Babcock, G.T., Vickery, L.E. and Palmer, G. (1976) J. Biol. Chem. 251, 7907–7919.
[27] Eaton, W.A. and Charney, E. (1969) J. Chem. Phys. 51, 4502–4505.