Apparent Adenosine Triphosphate Induced Ligand Change in Cytochrome a_3 of Pigeon Heart Mitochondria†

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ABSTRACT: Simultaneous oxidation–reduction potential *vs.* absorbance titrations have allowed us to make measurements of the individual spectral and thermodynamic properties of cytochromes *a* and a_3 in the presence or absence of added ligands. In uncoupled pigeon heart mitochondria, cytochrome a_3 accounts for approximately 60% of the total absorbance in the Soret band region (445–455-nm titrations) as compared to the almost equal contribution of the two components to the α band (605–630-nm titrations). The λ_{max} for cytochromes *a* and a_3 are not identical in either the α or Soret (γ) bands. In both cases the absorbance maximum of cytochrome a_3 is located at slightly shorter wavelengths than that for cytochrome *a*, *e.g.*, at 604 and 606 nm, respectively, in the α band. Addition of ATP to coupled pigeon heart mitochondria elicits a strong interaction between cytochromes *a* and a_3 which is expressed in the interdependence of their spectra and half-reduction potentials and is consistent with the occurrence of an energy-linked ligand change on the heme moiety of cytochrome a_3. The data are discussed in terms of the involvement of cytochrome a_3 in the primary energy transducing process at site III of the mitochondrial respiratory chain. The marked interdependence of the properties of cytochromes *a* and a_3 is also considered.

Isolated cytochrome oxidase has been shown to contain 2 moles of heme *a*, designated cytochromes *a* and a_3 by Keilin and Hartree (1939), and 2 moles of copper per mole of enzyme (Takemori, 1960; Griffiths and Wharton, 1961). Although the existence of cytochromes *a* and a_3 has been known for many years, all attempts to fractionate them and retain their activities in a native form have proved unsuccessful. Thus the spectral properties of these two components have been studied by utilizing the ability of one of the heme moieties (usually cytochrome a_3) to combine in either its oxidized or reduced form with various added ligands (Yonetani, 1960; Horie and Morrison, 1963; Slater *et al.*, 1965; Tzagoloff and Wharton, 1965; Vanneste, 1966). Such classical procedures indicate that approximately 50% of the absorbance of the Soret band (λ_{max} at 445 nm) of cytochrome oxidase is attributed to cytochrome a_3 while its contribution to the α band is much less, being only 11–28% of the total absorbance at 605 nm.

Cytochrome a_3 has therefore been routinely identified as the component which, in intact mitochondria or isolated cytochrome oxidase, will react with CN^- or CO. Calculations of this type, employing difference spectra to compute the individual contributions of the two species, make the assumption that no spectral interaction occurs between cytochromes *a* and a_3 in the presence of added ligands. Recently, however, Wilson *et al.* (1972b), by titrating the course of oxidation–reduction of cytochromes *a* and a_3 in the presence of redox mediators, have demonstrated that these two cytochromes contribute equally to the α band of cytochrome oxidase in the absence of added ligands. Addition of inhibitors, specific for cytochrome *a* or a_3 elicits a strong heme–heme interaction which is expressed in the interdependence of the absorption spectra and half-reduction potentials of these two components. Thus, in the presence of CO (specific for the reduced form of a_3), the extinction coefficient for cytochrome *a* in the α band is increased by more than 50% and its midpoint potential is altered by 30–40 mV to a more positive value.

Evidence for a possible copper–heme interaction has also been presented recently by King and Yong (1971), who consider that the optical absorbance spectrum of cytochrome a_3 may depend on the state of reduction of one of the copper atoms. The role of copper as a mediator in the heme–heme interaction process has also been considered by Wilson and coworkers (Wilson *et al.*, 1971; Wilson and Leigh, 1972).

This communication demonstrates that addition of ATP to coupled pigeon heart mitochondria elicits a strong heme–heme interaction which is consistent with the occurrence of a ligand change on the heme moiety of cytochrome a_3. This interaction is expressed in the interdependence of the half-reduction potentials and absorption spectra of cytochromes *a* and a_3. In the presence of ATP the low-potential component is identified as cytochrome a_3 by its reactivity with CO. A previous report (Wilson *et al.*, 1972a) has shown that ATP can promote an energy-linked spectral transformation in a ferricytochrome associated with the site III region and probably represents a high to low spin state transition in a component of cytochrome oxidase. It appears likely that the effects described in this paper reflect the consequences of this change for the properties of the reduced forms of cytochromes *a* and a_3.

Materials and Methods

Pigeon heart mitochondria were prepared by the method of Chance and Hagihara (1963). The procedures and equipment used to perform the oxidation–reduction potential *vs.* absorbance titrations have previously been described in detail (Dutton, 1970; Dutton *et al.*, 1970). Optical measurements were recorded with a dual-wavelength spectrophotometer designed and built in the Johnson Foundation. The bandwidth at half-height of the measuring light beams was always less than 1.5 nm.

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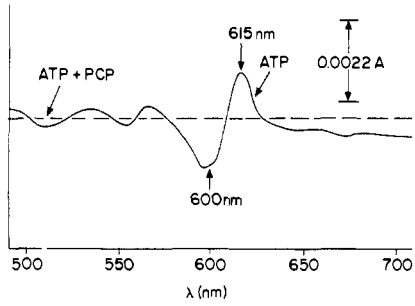

FIGURE 1: Energy-linked spectral alteration in the α band of cytochrome oxidase in pigeon heart mitochondria. Pigeon heart mitochondria (3.8 mg of protein/ml) were suspended in a medium containing 0.225 M mannitol, 0.05 M sucrose, and 0.05 M morpholinopropanesulfonate buffer (pH 7.2) (MS-MOPS, pH 7.2) in the presence of 5 mM glutamate, 5 mM succinate, 5 mM ascorbate, 2 μg/ml of antimycin A, 3 μM rotenone, and the redox mediators, diaminodurene (60 μM) and phenazine methosulfate (50 μM). The solid curve (+ATP) shows the spectral change induced by adding 3 mM ATP to the sample cuvet. A base line was obtained after addition of 5 μM pentachlorophenol or 2 μg/ml of oligomycin, both of which eliminate the ATP-induced change. Spectral alterations were recorded using an Aminco-Chance dual-wavelength, split-beam spectrophotometer and a measuring spectral bandwidth at half-height of 1 nm.

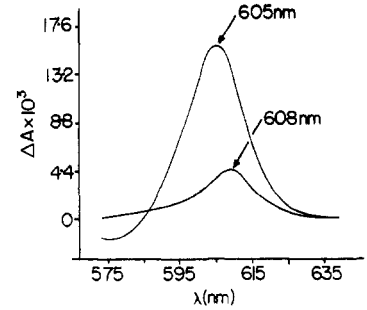

FIGURE 2: The spectrum of reduced cytochrome a_3 in the α band in the presence of ATP. A conventional oxidized–reduced spectrum for the α band of cytochrome oxidase (λ_{max} at 605 nm) is presented using pigeon heart mitochondria (3.5 mg of protein/ml) reduced with 5 mM malate plus glutamate as substrates in MS-MOPS buffer (pH 7.2). After measuring the above spectrum on an Aminco-Chance dual-wavelength split-beam spectrophotometer, the mitochondria in the reference cuvet were reduced with 5 mM malate plus glutamate also. Addition of 6 mM ATP to this cuvet yields the difference spectrum with an absorbance maximum at 608 nm (lower trace). The base line was taken after the energy-linked oxidation was abolished with 2 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

The reagents used were the same as described previously (Dutton *et al.*, 1970) and routine chemicals were of the highest grade available commercially.

Results

Energy-Linked Spectral Alteration in the α Band of Cytochrome Oxidase. Addition of ATP to an anaerobic suspension of pigeon heart mitochondria, maintained in a highly reduced state, in the presence of succinate, glutamate, ascorbate, antimycin A, rotenone, and the redox mediators diaminodurene and phenazine methosulfate, results in the occurrence of a small spectral shift in the α band of cytochrome oxidase (Figure 1). This energy-linked spectral alteration is to longer wavelengths and is characterized by a λ_{min} at 600 nm and a λ_{max} at 615 nm with an isosbestic point at approximately 608 nm. The reaction is sensitive to oligomycin or uncouplers and is stable for 10–15 min at room temperature until ATP hydrolysis lowers the phosphate potential sufficiently to reverse the shift. The conditions of the experiment eliminate the possibility that this spectral change is caused by an ATP-induced oxidation–reduction of cytochromes *a* and a_3. It can be seen that the presence of redox mediators has prevented to a great extent the energy-linked oxidation of *b* cytochromes which is normally seen in antimycin A inhibited mitochondria. A similar ATP-induced alteration in the α band of cytochrome oxidase has been previously reported by Wikström and Saris (1967) who attributed the effect to a change in the reduced spectrum of cytochrome a_3.

Further evidence on the origin of this energy-linked spectral change in cytochrome oxidase is presented in Figure 2. A difference spectrum is revealed which utilizes the observations of Wilson and Chance (1967) and Bonner and Plesnicar (1967) that cytochrome a_3 is not maintained in a highly reduced state (in anaerobic mitochondria) under conditions of high phosphate potential. Thus a "crossover" can be induced between cytochromes *a* and a_3 in which cytochrome a_3 is highly oxidized and cytochrome *a* remains essentially reduced.

Accordingly pigeon heart mitochondria were allowed to become anaerobic in an open cuvet using malate plus glutamate as substrates. Addition of ATP to the reference cuvet gives the difference spectrum shown in Figure 2 and a conventional oxidized–reduced spectrum for the α band of cytochrome oxidase with a λ_{max} at 605 nm is included for comparison. The (reduced) − (reduced + ATP) spectrum represents the difference spectrum of reduced cytochrome a_3 in the presence of ATP. It has a λ_{max} at 608 nm and the size of the peak is about 20% of the oxidized–reduced peak for the α band of cytochrome oxidase. This energy-linked oxidation of cytochrome a_3 can be reversed by oligomycin or uncouplers and can also be eliminated by the addition of the redox mediators, phenazine methosulfate and diaminodurene. The long-wavelength nature of this peak suggests that the energy-linked shift demonstrated in Figure 1 may be the result of an ATP-induced change in the spectrum of reduced cytochrome a_3.

ATP Effects on the Course of Oxidation–Reduction of Cytochromes a and a_3 in the α Band of Pigeon Heart Mitochondria. Previous attempts to resolve the Soret band titrations at pH 7.2 into two components at high phosphate potentials have proved unsuccessful (Wilson *et al.*, 1971). However, the evidence indicated that under these conditions, cytochrome a_3 could attain a midpoint potential more negative than cytochrome *a* while the latter component did not appear to be markedly affected by addition of ATP. This difficulty has now been resolved by performing the titrations in the presence of antimycin A and close agreement has been achieved between Soret and α-band titrations.

Using the wavelength pair 445–455 nm, the course of oxidation–reduction of the *a*-type cytochromes was influenced by a small contribution from cytochrome b_T (λ_{max} at 430 nm) which, in the presence of ATP, has an $E_{m7.2}$[1] of +240 mV and is thus reduced over the same potential range as cytochromes *a* and a_3. The discovery of Dutton *et al.* (1972), that formation of the ATP-induced "high-energy" form of cytochrome b_T is inhibited by antimycin A, has allowed us to resolve energy-

[1] Abbreviations used are: $E_{m7.2}$ is the midpoint potential of an oxidation–reduction couple at pH 7.2; *n* is the number of electrons transferred in the oxidation–reduction reaction. FCCP is carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

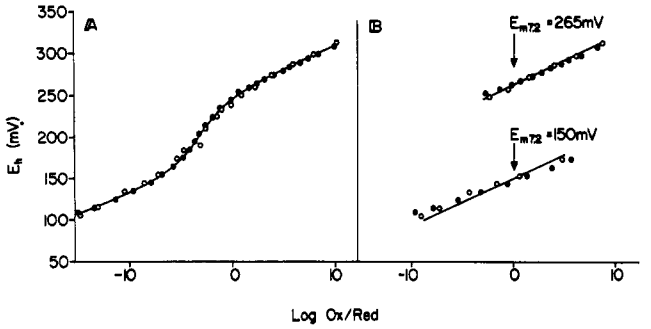

FIGURE 3: The course of oxidation–reduction of cytochromes *a* and a_3 in the α band of cytochrome oxidase in the presence of 6 mM ATP. Pigeon heart mitochondria (2.4 mg of protein/ml) were suspended under an atmosphere of argon (1 ppm of O_2) in MS-MOPS buffer (pH 7.2). Diaminodurene (80 μM) and phenazine methosulfate (60 μM) were employed as redox mediators. Other additions were antimycin A (2 μg/ml) and ATP (6 mM). The measuring wavelengths were 605–630 nm. Part A reveals the logarithm of the absorbance of the oxidized to reduced cytochrome plotted as a function of the redox potential change from 380 to 80 mV. Both oxidative (○) and reductive (●) titrations were performed. In part B the curve is resolved arithmetically into two components; theoretical $n = 1$ lines are drawn through the points.

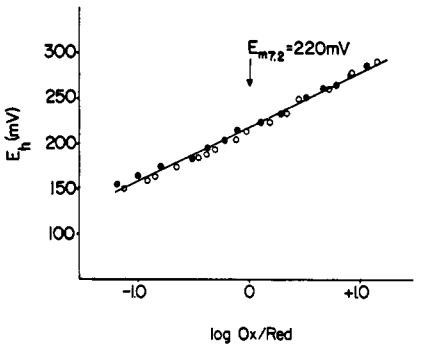

FIGURE 4: The effect of CO on the course of oxidation–reduction of cytochromes *a* and a_3 in the presence of 6 mM ATP. Pigeon heart mitochondria (2.2 mg of protein/ml) were maintained under the conditions of Figure 3 except that the medium was saturated with CO (2 mM). Both oxidative (○) and reductive (●) titrations were performed and a theoretical $n = 1$ line was drawn through the experimental points.

linked effects at the site III region of the respiratory chain in the absence of interference from site II.

It has also been found necessary to include antimycin A during *a* and a_3 titrations in the α band (605–630 nm); otherwise a *net* oxidation is normally seen as the redox potential of the system is lowered from 300 to 200 mV. This phenomenon may be related to reduction of the ATP-induced ferricytochrome peak at 577 nm over this potential range (Wilson *et al.*, 1972a) or again be the result of interference from the site II region caused by reduction of cytochrome b_T. Antimycin A has no effect on the course of oxidation–reduction of cytochromes *a* and a_3 in the uncoupled state.

In Figure 3A the course of oxidation–reduction of cytochromes *a* and a_3 (605–630 nm) is presented in the presence of 6 mM ATP. The sigmoid character of the curve is as expected for two components with quite different half-reduction potentials. Arithmetic resolution of the two species (Figure 3B) yields two components with *n* values of 1 and $E_{m7.2}$ of 265 and 150 mV, respectively. The high-potential component accounts for 70–75% and the low-potential component 25–30% of the total absorbance change at these wavelengths. Confirmation of these values has been achieved by repeating the titrations in the Soret band using the wavelength pair 445–455 nm. Over many experiments the measured midpoints of the 2 components varied from 260 to 285 mV and from 145 to 165 mV, respectively. Similar results could be obtained in coupled pigeon heart mitochondria in which 90–95% of the cytochrome *c* had been extracted as determined by spectral analysis at liquid nitrogen temperatures. Under these conditions, the response of cytochromes *a* and a_3 to the addition of small amounts of oxidant or reductant was noticeably slower, consistent with the view that cytochrome *c* is the principal site of interaction for the redox mediators. In addition, the observed midpoint potential changes were ATP specific and could be reversed by suitable concentrations of ADP and P_i as well as by oligomycin or uncouplers. The phosphate potential dependence of the spectral and thermodynamic alterations in cytochromes *a* and a_3 is currently under active investigation.

In uncoupled mitochondria, two species have been shown to contribute almost equally to the absorbance change at 605–630 nm, while in the Soret band cytochrome a_3 accounts for about 60% of the total absorbance using the wavelength pair 445–455 nm. Close agreement is obtained for the midpoint potentials of cytochromes a_3 and *a*, being 375 ± 10 and 220 ± 10 mV at pH 7.2, respectively.

In view of the complex spectral and midpoint potential interactions which are revealed by the ATP titrations, it becomes necessary to ascertain which of the two species, observed in the presence of ATP, can be identified as cytochrome a_3. Accordingly, a standard titration (605–630 nm) was performed in the presence of 6 mM ATP and saturating amounts of CO (Figure 4). The effect of CO (an inhibitor specific for the reduced form of cytochrome a_3) is to completely remove the 150-mV species from the titration using this wavelength pair and only one component remains which titrates as an $n = 1$ acceptor with an $E_{m7.2}$ of 220 mV. Under the conditions of the titration (300 → 150 mV) the a_3^{2+}–CO compound is unchanged and can be detected by its characteristic absorption maximum at 590 nm. Thus the low-potential component is identified as cytochrome a_3 by its reactivity with CO.

If the titration in Figure 4 is repeated after the addition of 2 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, cytochrome *a* is now titrated with an $E_{m7.2}$ of 260 mV in agreement with the value reported by Wilson *et al.* (1972b). This would indicate that ATP is able to induce small changes in the midpoint potential of cytochrome *a* even in the presence of CO.

The action of CO and ATP in modifying the midpoint potential of cytochrome *a* is examined in Figure 5. If coupled pigeon heart mitochondria are maintained under strictly anaerobic conditions (saturating CO present) at a redox potential of 250 mV, cytochrome *a* is only half-reduced while cytochrome a_3 is present as the a_3^{2+}–CO compound which causes no interference with our measurements at 605–630 nm. Addition of ATP produces an oxidation of cytochrome *a* equivalent to about 28% of its total absorbance at these wavelengths. This oxidation is reversed by addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Since we have previously measured the $E_{m7.2}$ of cytochrome *a* in the presence and absence of ATP (CO present) to be 220 and 260 mV, respectively, it can be calculated (see Clark, 1960) that addition of ATP at 250 mV should cause a 31% oxidation of cytochrome *a*, in close agreement with the experimentally observed value. Any possibility that the measured changes are due to an ATP-

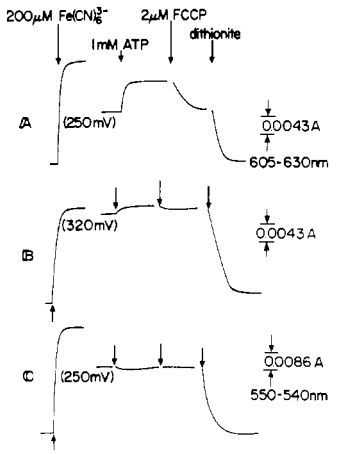

FIGURE 5: The effect of ATP on the oxidation–reduction midpoint potential of cytochrome *a* in the presence of CO. The experimental procedures were as described in Figures 3 and 4 except that pigeon heart mitochondria were present at 2.5 mg of protein/ml. Additions were made as indicated on the figure. ATP solutions were bubbled with argon to remove traces of oxygen. The measuring wavelengths employed for cytochrome *a* were 605–630 nm (traces A and B) and 550–540 nm for cytochrome *c* (trace C). An upward deflection in the trace indicates oxidation.

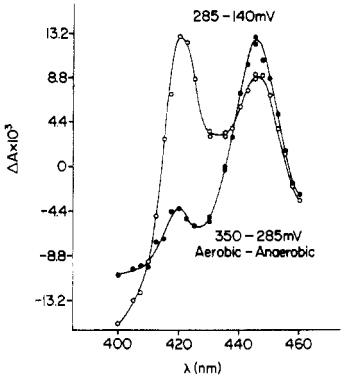

FIGURE 6: Spectra of cytochromes *a*, a_3, and *c* in the Soret band in the absence of added ligands. Pigeon heart mitochondria (0.55 mg of protein/ml) were employed in the same system as described in Figure 3 except that the redox mediators employed were diaminodurene (40 μM) and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (30 μM). The total absorbance change from 350 mV (aerobic) to 285 mV (anaerobic) represents principally cytochrome a_3 (●) with $E_{m7.2}$ of 375 mV and that from 285 to 140 mV (○) represents cytochrome *a* ($E_{m7.2}$ of 220 mV). These absorbance values are plotted as a function of wavelength from 400 to 455 nm. Each point is the average of two measurements taken during reduction with NADH and a subsequent oxidation of cytochromes *a* and a_3. Since ferricyanide interferes very badly in this spectral region, reoxidation was accomplished by introducing small amounts of oxygen into the cuvet and allowing the sample to become anaerobic once more in the desired potential range.

induced oxidation of cytochrome a_3 is eliminated by repeating the experiment at 320 mV at which potential no significant ATP-induced effects can be detected. Such changes would be expected to be considerably enhanced as the redox potential of the system approaches the midpoint potential for that component. Analysis of cytochrome *c* ($E_{m7.2}$ of 235 mV), with the system poised at 250 mV, fails to detect any ATP-induced effects on the state of reduction of this component, providing good evidence that no energy-linked alteration occurs in the midpoint potential of cytochrome *c*. Previous investigations have already noted that the midpoint potential of cytochrome *c* is independent of the "energy state" of the mitochondria (Hinkle and Mitchell, 1970; Dutton *et al.*, 1970).

The former authors also noted a lowering (40–50 mV) in the midpoint of cytochrome *a* (and a_3) in CO-inhibited rat liver mitochondria after addition of ATP. They attributed this change to the influence of an ATP-induced membrane potential which might tend to cause oxidation of any component not in direct electrical contact with the outer aqueous phase. However, CO alone will increase the midpoint of cytochrome *a* by some 40 mV (Wilson *et al.*, 1972b). Moreover, the midpoint of cytochrome *a* will increase or decrease by about 40 mV on addition of ATP depending on whether CO is present initially as shown in Figures 3, 4, and 5. These results appear to be consistent with the existence of a close interaction between cytochromes *a* and a_3 while they are not readily interpreted in terms of a "membrane potential" mechanism.

Analysis of ATP Effects on the Spectra of Cytochromes a and a_3 as Separated on a Potential Basis. A spectral analysis of cytochromes *a*, a_3, and *c* in the Soret region in the absence of added ligands is presented in Figure 6. The individual contributions of the cytochromes are obtained by measuring the absorbance changes from 350 (aerobic) to 285 mV (anaerobic) and from 285 to 140 mV as a function of wavelength. The spectra are consistent with titration measurements which indicate that cytochrome a_3 contributes about 60% of the total absorbance in the Soret band of cytochrome oxidase. The absorbance band at 420 nm is attributable to cytochrome *c* which has an $E_{m7.2}$ of 235 mV and is thus reduced over the same potential range as cytochrome *a*. The spectrum of cytochrome a_3 also contains a small contribution from cytochrome *c* since the potential cut is taken at 285 mV, where cytochrome *c* is 10–20% reduced. There is no interference from either cytochrome b_K ($E_{m7.2}$ of +30 mV) or cytochrome b_T ($E_{m7.2}$ of −30 mV) in these spectra.

The individual spectra for cytochromes *a* and a_3 in the α band have been published previously (Wilson *et al.*, 1972b) and establish the almost equal contribution of the two cytochromes to the 605-nm absorbance maximum in the absence of added ligands. Our previous data also indicated the λ_{max} for cytochrome a_3 may be at slightly shorter wavelengths (604 nm) than the peak for cytochrome *a* (606 nm). The effect of ATP on the spectra of cytochromes *a* and a_3 in the α band is demonstrated in Figure 7. It is immediately apparent that there has been a dramatic change in the relative contribution of the two species to the total absorbance. The 265-mV component (350–200 mV) now accounts for about 70% of the absorbance and has a λ_{max} at 604–605 nm. The minor component with $E_{m7.2}$ at 150 mV (200–85 mV; identified as cytochrome a_3) now appears to have a λ_{max} at approximately 608 nm, slightly longer than cytochrome *a*.

In an attempt to analyze more precisely the origin of the ATP-induced spectral alteration (Figure 1) and to verify the apparent spectral shift in cytochrome a_3 (Figures 2 and 7), titrations were performed using the wavelength pair 602–608 nm, *i.e.*, the wavelength pair which is symmetrically distributed about the 605-nm absorbance peak for cytochrome oxidase. Such procedures (assuming symmetrical peaks) provide a very sensitive indicator for detection of slight differences in the absorbance maxima of the individual species. Thus if both cytochromes *a* and a_3 have identical peaks at 605 nm no significant absorbance changes will be noted as these components are reduced. However, any deviation of their λ_{max} to shorter or longer wavelengths will be detected as a *net* increase in absorbance at 602 or 608 nm, respectively.

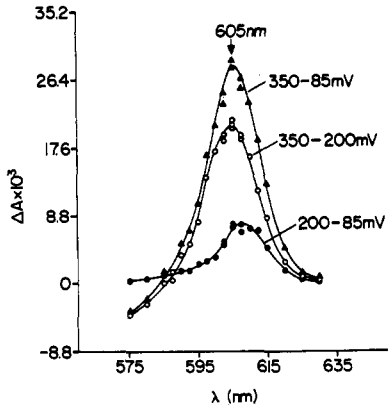

FIGURE 7: Spectra of cytochromes *a* and a_3 in the α band in the presence of ATP. The experimental procedures were as described in Figure 3. Pigeon heart mitochondria were present at 2.6 mg of protein/ml. Absorbance changes were recorded as a function of wavelength over the entire α band of cytochrome oxidase in the potential range 350–200 mV (○) and from 200 to 85 mV (●). These changes represent principally cytochrome *a* with $E_{m7.2}$ of 265 mV and cytochrome a_3 with $E_{m7.2}$ of 155 mV under these conditions. The total absorbance change from 350 to 85 mV (▲) showing the conventional α band for cytochrome oxidase is included for comparison. Each point is the average of two measurements taken during a reductive titration with NADH and subsequent reoxidation with potassium ferricyanide.

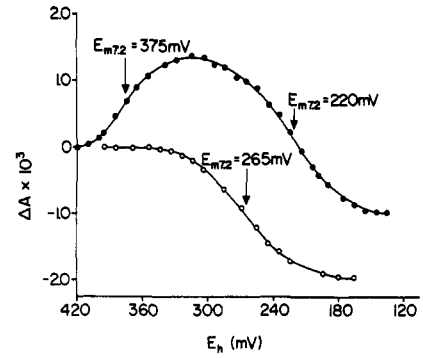

FIGURE 8: The course of oxidation–reduction of cytochromes *a* and a_3 in pigeon heart mitochondria and the effects of CO as revealed by titrations at 602–608 nm. A reductive titration (●) was performed using the general conditions described in Figure 3. Pigeon heart mitochondria were present at 1.8 mg of protein/ml and no antimycin A or ATP was added. The measuring wavelengths were 602–608 nm. The experiment was repeated after saturating the anaerobic mitochondrial suspension with CO (○). An upward trend in the titration represents a *net* increase in absorbance at 602 nm.

The results of such an experiment are shown in Figure 8. Over the potential range that cytochrome a_3 is known to be reduced, a *net* increase in absorbance at 602 nm is observed. The course of this oxidation–reduction indicates that the component is a one-electron acceptor ($n = 1$) with an $E_{m7.2}$ of 375 mV, identical with the previously obtained values for cytochrome a_3. As the titration is continued over the potential range 260–150 mV a *net* absorbance increase at 608 nm is observed, suggesting that cytochrome *a* has a λ_{max} at slightly longer than 605 nm. It titrates as an $n = 1$ species with an $E_{m7.2}$ of 220 mV. It should be noted that these data are consistent with the spectral analysis presented previously (Wilson *et al.*, 1972b) which indicated that cytochromes *a* and a_3 have slightly different reduced peaks located on opposite sides of the 605-nm maximum for cytochrome oxidase.

Dye controls, performed without mitochondria, show that these small absorbance changes are not the result of interference from added redox mediators. Further confirmation is achieved by repeating that titration in the presence of saturating amounts of CO (Figure 8). This inhibitor combines specifically with the reduced form of cytochrome a_3 and raises its midpoint potential (according to the equations described by Clark, 1960) such that it can no longer be oxidized by ferricyanide and thus it is effectively removed from the titration. It can be seen that no absorbance change due to cytochrome a_3 is detected in the presence of CO while cytochrome *a* is reduced with a slightly altered midpoint potential ($E_{m7.2}$ of 265 mV), a manifestation of CO-induced heme–heme interaction (Wilson *et al.*, 1972b). Thus the validity of this procedure for the measurement of cytochromes *a* and a_3 is confirmed from a direct estimate of their *n* values and midpoint potentials and from their responses to CO.

A precise estimate of the absorbance maxima for the individual reduced spectra of cytochromes *a* and a_3 can be achieved by extending this technique to perform titrations at a series of wavelength pairs (600–606, 601–607, ... 606–612 nm) covering the α band of cytochrome oxidase. If the point of symmetry of a given wavelength pair (*e.g.*, 603 nm for the wavelength pair 600–606 nm) is shorter than the λ_{max} for a given species a *net* absorbance increase at 606 nm will be observed as this component is reduced. As the λ_{max} of this component is approached (using different wavelength pairs) the absorbance change will decrease until its λ_{max} and the point of symmetry of the measuring wavelength pair correspond at which stage no *net* change will be observed. As we continue to longer wavelength pairs the absorbance changes for that component will now be in the opposite direction. Thus we obtain a series of titration curves which completely define the λ_{max} for cytochromes *a* and a_3 in uncoupled mitochondria (Figure 9). In the case of cytochrome *a* no *net* change is observed using the wavelengths 603–609 nm indicating that its absorbance maximum is at 606 nm. For cytochrome a_3 the measured λ_{max} is at approximately 603 nm.

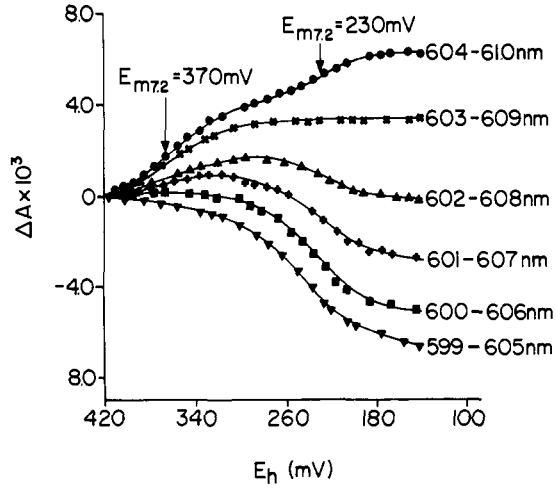

FIGURE 9: Determination of the individual absorbance maxima of cytochromes *a* and a_3 in the α band of cytochrome oxidase. Titrations were performed at a series of wavelength pairs covering the α band of cytochrome oxidase as indicated in the figure; otherwise identical conditions were employed to those described in Figure 3. Pigeon heart mitochondria were suspended at a final concentration of 2.0 mg of protein/ml. An upward deflection in the trace indicates a *net* absorbance increase at the shorter of a given wavelength pair.

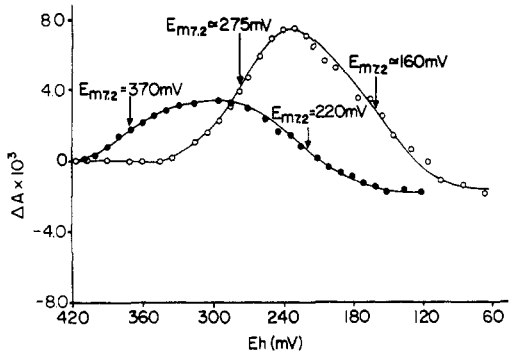

FIGURE 10: The effects of ATP on the course of oxidation–reduction of cytochromes *a* and a_3 as revealed by titrations at 602–608 nm. Pigeon heart mitochondria (3.5 mg/ml) were used to perform a reductive titration (602–608 nm) in the presence of 6 mM ATP (○) under the conditions detailed in Figure 3. The lower trace (●) was obtained by repeating the experiment after addition of 2 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

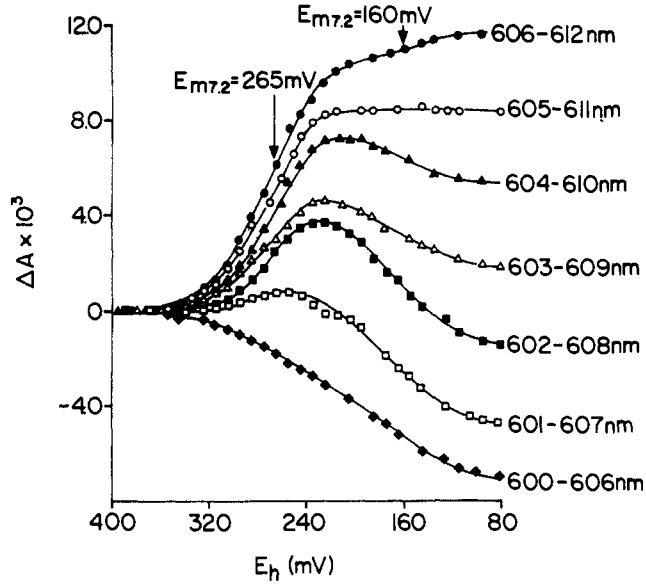

FIGURE 11: Determination of the individual absorbance maxima of cytochromes *a* and a_3 in the α band of cytochrome oxidase in the presence of ATP. A series of reductive titrations were performed on pigeon heart mitochondria (2.0 mg of protein/ml) after addition of 2 μg/ml of antimycin A and 5 mM ATP. The wavelength pairs employed were as indicated on the figure; other experimental procedures were as described in Figure 3. An *net* increase in absorbance at the shorter of a given wavelength pair is represented by an upward trend in the course of the reductive titration.

However, since the spectrum of cytochrome a_3 has a shoulder at shorter wavelengths (Wilson *et al.*, 1972b) the λ_{max} may be nearer 604 nm. Throughout these titrations cytochromes *a* and a_3 are readily identified on a potential basis as their respective midpoint potentials are separated by some 150 mV.

Analysis of ATP Effects on the Spectra of Cytochromes a and a_3 by Titrating at a Series of Wavelength Pairs. In Figure 10 the effect of ATP on the titration at 602–608 nm is demonstrated and the uncoupled situation is included for comparison. Two components are again readily observed which have spectral peaks located on opposite sides of 605 nm. Both species titrate as one electron acceptors and have midpoint potentials ($E_{m7.2}$ of 275 and 160 mV) which closely correspond to those for cytochromes *a* and a_3 as revealed by the standard titration (Figure 3). There is a degree of error in these estimates since the midpoint potentials of the two species are separated by only 100 mV. Thus there is a 10–15% overlap in the oxidation–reduction curves for the separate components which will have the effect of increasing slightly the measured value for the $E_{m7.2}$ of the high-potential component while causing a small apparent decrease in the $E_{m7.2}$ of the low potential species.

The most striking feature of the ATP titration is that the absorbance changes observed are approximately two times larger than is found in the uncoupled situation indicating that ATP-induced spectral changes have occurred in both components. The results would suggest that the two components now have α bands which are more asymmetrically distributed about the 605-nm peak for cytochrome oxidase. Thus the 275-mV species has a λ_{max} at less than 605 nm while that of the low-potential component (160 mV) is longer than 605 nm.

Since we have previously identified the 160-mV species as cytochrome a_3, it is evident that its λ_{max} has now shifted to longer wavelengths. In the uncoupled situation, the absorbance peak for cytochrome a_3 was estimated to be 604 nm whereas, in this case, the measured absorbance changes for cytochrome a_3 are in the opposite direction indicating that its λ_{max} is now located at longer than 605 nm. Surprisingly, the data also provide evidence for a spectral change to shorter wavelengths in cytochrome *a* which now appears to have a peak situated below 605 nm.

A precise quantitation of the size of the spectral shifts of cytochromes *a* and a_3 is achieved by once more titrating at a series of wavelengths pairs (Figure 11). In this manner, the λ_{max} for cytochrome a_3, under these conditions, is found to be at 608 nm, in close agreement with the spectral data in Figures 2 and 7. In the presence of ATP, the peak for cytochrome *a* is measured at 604 nm. Thus it must be concluded that ATP can produce a 4- to 5-nm red shift in the reduced peak of cytochrome a_3. At the same time, however, the spectrum of cytochrome *a* is also modified such that it now absorbs maximally at 604 nm as compared to 606 nm in the uncoupled state.

Discussion

In this communication we have extended our original observations on cytochrome oxidase in pigeon heart mitochondria to include the existence of an ATP-induced heme–heme interaction which is consistent with the primary event being an energy-linked ligand change on the heme moiety of cytochrome a_3. This alteration is manifested by both midpoint potential and spectral changes in the reduced form of cytochrome a_3 supporting the concept that this cytochrome is involved in the primary energy transduction process at site III. The situation is complicated, however, by the secondary effects of heme–heme interaction which are expressed as changes in the relative contributions of cytochromes *a* and a_3 to the α and Soret bands of cytochrome oxidase and as small modifications in the spectra and half-reduction potential of cytochrome *a*. The individual spectra of cytochromes *a* and a_3 also appear to be dependent on the state of reduction of their companion component.

Spectra of Cytochromes a and a_3 in the Absence of Added Ligands. Analysis of the individual spectra of cytochromes *a* and a_3 in the Soret band as separated on a potential basis indicates that approximately 60% of the absorbance at 445

nm is contributed by cytochrome a_3 and 40% by cytochrome *a*. These results are in close agreement with the classical data utilizing difference spectra techniques with CO and CN^- to separate the spectra of these two components.

The situation in the α band indicates that cytochromes *a* and a_3 contribute almost equally to the total absorbance at 605 nm as separated on a potential basis. These results are compatible with the classical data when heme–heme interaction is taken into account as discussed previously (Wilson *et al.*, 1972b). It is not clear why the α and Soret bands of cytochrome oxidase should respond so differently to the action of added ligands such as CO or CN^- since, in the unliganded case, the contribution of cytochromes *a* and a_3 to these two bands appears to be very similar. However, the influence of copper in modifying the absorbance spectra of cytochromes *a* and a_3 in the presence or absence of added ligands is still obscure. The recent work of King and Yong (1971) is indicative that the observed heme–heme interactions may be mediated *via* one atom of copper. At present we have no evidence for or against the participation of copper in the heme–heme interaction or energy coupling mechanisms.

Titrations, using the wavelength pair 602–608 nm (Figure 8) confirm our previous spectral data indicating that cytochrome a_3 has a slightly differing λ_{max} from cytochrome *a*. Thus the λ_{max} for cytochrome a_3 is shorter than 605 nm (at 604 nm) and for cytochrome *a* longer than 605 nm (at 606 nm) in the uncoupled system. A completely analogous situation is observed in the Soret band (442–448-nm titrations). Cytochrome a_3 is found to be the short-wavelength component and cytochromes *a* and a_3 have individual absorbance maxima located on opposite sides of the 445-nm absorbance band for cytochrome oxidase.

ATP-Induced Midpoint Potential Change in Cytochrome a_3. Titrations of cytochrome oxidase in pigeon heart mitochondria at 445–455 or 605–630 nm in the presence of ATP reveal the presence of two components with $E_{m7.2}$ of 275 ± 10 and 155 ± 10 mV, respectively. In both cases the relative contribution of the two species to the total absorbance is 70–75 and 25–30%, respectively, as compared to the almost equal contribution in the uncoupled state. The situation in the α band is analogous to the binding of CO to cytochrome a_3 under which conditions the contribution of cytochrome *a* to the total absorbance is increased by more than 50% and its midpoint potential is also shifted to more positive values by some 40 mV. This would suggest that the 265-mV species in the presence of ATP is cytochrome *a* and this is confirmed by repeating the experiment after addition of CO. Under these conditions the low-potential component is identified as cytochrome a_3 by its reactivity with CO when it is completely removed from the titration (Figure 4). Thus at high phosphate potentials the $E_{m7.2}$ of cytochrome a_3 is altered by approximately 220 mV consistent with the view that it is the primary energy transducing component at site III of the mitochondrial respiratory chain.

ATP-Induced Spectral Alterations in Cytochrome a_3. The ATP-induced change in the contributions of cytochromes *a* and a_3 to the α band is confirmed in the spectra shown in Figure 7 which demonstrate that, under these conditions, cytochrome a_3 (low-potential species) is the minor component. Several lines of evidence also indicate the long-wavelength nature of cytochrome a_3 (λ_{max} at 608 nm) at high phosphate potential, *i.e.*, a 4- to 5-nm shift to longer wavelengths occurs on addition of ATP. One of the predictions arising from the observed midpoint potentials changes on cytochromes b_T and a_3 is that these cytochromes have more than one oxidized or reduced species (or both) and that the interconversion of these forms is energy dependent. Thus we have direct evidence for the existence of two reduced forms of cytochrome a_3, the "high-energy" species having a λ_{max} at 608 nm. Previous data (Wilson *et al.*, 1972a) have indicated that an energy-linked spectral transformation takes place in an oxidized component of cytochrome oxidase, consistent with a high- to low-spin transition in a hemoprotein at site III of the mitochondrial respiratory chain. The addition of ATP to mitochondria also decreases the rate of reaction of cyanide with oxidized cytochrome oxidase by a factor of at least 10^3 (Erecinska *et al.*, 1972). Therefore it may be that cytochrome a_3 additionally has two oxidized forms differing in the ligand binding to the iron atom and in their reactivity with cyanide. Since, in many respects, the ATP effects on the reduced forms of cytochromes *a* and a_3, closely resemble the situation after binding of CO, suggesting the occurrence of an ATP-induced ligand change on cytochrome a_3, it appears that both oxidized and reduced forms of cytochrome a_3 may exist in high-energy states which are dependent on the prevailing phosphate potential. The results presented in this communication probably reflect the consequences of the spin state transition, observed in the oxidized species, for the spectral and thermodynamic properties of the reduced forms of cytochromes *a* and a_3.

ATP-Induced Alterations in the Spectrum and Midpoint Potential of Cytochrome a. The concept of cytochrome oxidase which emerges from these experiments is that cytochromes *a* and a_3 cannot be regarded as separate entities but are, in fact, two closely related, interacting species. Therefore, compounds which markedly affect the properties of cytochrome a_3, *e.g.*, CO and ATP, also exert an influence on the midpoint potential and spectral characteristics of cytochrome *a*. In this respect, addition of ATP or saturating levels of CO increase the $E_{m7.2}$ of cytochrome *a* by 40–50 mV. However, with both CO and ATP present, the half-reduction potential of cytochrome *a* is 220 mV, its normal value in untreated pigeon heart mitochondria. The ATP effects on the midpoint potential of cytochrome *a*, in the presence or absence of CO, are reversed by addition of uncoupler (Figure 5).

The employment of redox mediators in Figure 5 gives rise to a "potential clamped" system in which the membrane-bound cytochromes are in rapid equilibrium with the added dyes. Thus the ATP-induced change in the E_m of cytochrome *a* can be attributed to an alteration in the chemistry of this component while it is obvious that no similar change occurs in the E_m of cytochrome *c*. The potential clamp technique, where the system is poised at a given redox potential under equilibrium conditions, can be usefully employed to detect the respiratory chain components whose midpoint potentials are affected by the "energy state" of the mitochondria.

The idea of a cytochrome *a*, modified by heme–heme interactions, is supported by an analysis of the spectral changes associated with the addition of CO or ATP to pigeon heart mitochondria. The presence of CO does not result in a marked alteration in the absorbance maximum of cytochrome *a* (as measured by 602–608-nm titrations) which still appears to be located at approximately 606 nm under these conditions. However CO produces a large increase in the extinction coefficient of cytochrome *a* which would be expected to cause an increase in the absorbance contribution of cytochrome *a* to the 602–608-nm titration, provided no broadening or shift in peak position has occurred. Since, in several such experiments, the absorbance change of cytochrome *a* (CO present) was diminished by 25–50%, a slight spectral shift to

TABLE I: Summary of the Effects of CO and ATP on the Spectra and Midpoint Potentials of Cytochromes *a* and a_3 as Measured by the Oxidation–Reduction Potential *vs.* Absorbance Technique in Intact Pigeon Heart Mitochondria.

	No ATP		+ATP		+CO	
Cytochrome	*a*	a_3	*a*	a_3	*a*	a_3
α-band maximum (nm)	606	604	604	608	606	590
Per cent contribution 605–630 nm	50	50	70–75	25–30	85[a]	
Per cent contribution 445–455 nm	40	60	70–75	25–30	40[a]	
$E_{m7.2}$ α or β band	220 ± 10	375 ± 10	275 ± 10	155 ± 10	255 ± 10	~560[b]

[a] Expressed as per cent of the control in the absence of CO. [b] Value is calculated assuming medium is saturated with CO (2 mM).

shorter wavelengths still remains a possibility. This shift, however, is certainly less than 1 nm.

A more dramatic change in the absorbance maximum of cytochrome *a* is produced by addition of ATP. The extinction coefficient is again increased in a directly analogous manner to the CO-induced effects. However, in this case cytochrome *a* assumes a λ_{max} at 604 nm as compared to 606 nm in uncoupled mitochondria.

In some respects the ATP situation is unique in that the spectrum of reduced *a* is obtained while cytochrome a_3 remains fully oxidized. Thus the observed spectral change on addition of ATP may represent a dependence of the absorption spectrum of cytochrome *a* on the state of reduction of cytochrome a_3. The involvement of copper in this process cannot be excluded and changes in the oxidation–reduction state of this component may also influence the spectra of the two hemoproteins (Greenwood and Gibson, 1967). Similarly, the ATP-induced alteration in the reduced spectrum of cytochrome a_3 may be modified by the fact that it is measured under conditions where cytochrome *a* is already reduced. Thus it is difficult to estimate the extent to which the observed spectral alteration in cytochrome a_3 can be attributed directly to an energy-linked change in this component and how this may be influenced by the oxidation–reduction state of cytochrome *a* or copper.

Nature of Cytochrome Oxidase. The results presented in this paper lend support to the original concept of cytochrome oxidase as described by Keilin and Hartree (1939) who suggested that cytochromes *a* and a_3 are closely interacting if not interconvertible species, and the "Siamese twins" hypothesis of King *et al.* (1965) which stresses the mutual dependence of these two components. The cooperativity between the heme groups demonstrates clearly their proximity in the native cytochrome oxidase complex, although it may be that the individual hemes are located in separate but closely interacting polypeptide chains as in the α and β subunits of hemoglobin.

The requirement for copper in the synthesis and assembly of cytochrome oxidase has been well established (Wohlrab and Jacobs, 1967) indicating the essential nature of this component for the normal functioning of the oxidase. Under conditions where synthesis of cytochrome oxidase is limited by the availability of copper, the stoichiometry between cytochromes *a* and a_3 is always maintained (Keyhani and Chance, 1971), suggesting that assembly of this macromolecular unit is a highly integrated process. In view of the close spectral and thermodynamic interactions observed in these studies, it appears that the unique spectral properties of *a* and a_3 and their characteristic reactivity to added ligands are associated with their presence in the intact oxidase complex. Physical separation of these components destroys these interactions and presumably alters the properties of the constituent hemoproteins such that the individual species are no longer recognizable.

A summary of the CO- and ATP-induced effects on the spectra and midpoint potentials of cytochromes *a* and a_3 as measured *in situ* using the oxidation–reduction potential *vs.* absorbance technique is presented in Table I. The data provide additional evidence for the intimate involvement of cytochrome a_3 in the energy transduction mechanism at site III of the mitochondrial respiratory chain. Alteration of the electronic configuration of the heme group of cytochrome a_3 by addition of ligands or by changing its oxidation–reduction state leads to modifications in the spectra and half-reduction potential of cytochrome *a*. The role of copper in the energy-coupling and heme–heme interaction processes is a subject for further experimentation.

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Fb, a New Enzymatic Fragment of Human γG Immunoglobulin†

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ABSTRACT: Extended digestion of human γG immunoglobulin with subtilisin (EC 3.4.4.16) produces several large fragments and a mixture of small peptides. Chemical and immunological characterization of the two principal fragments indicates that one, Fb(s), is composed of the C_L and C_H1 homology regions of the molecule and that the other, Fc′(s), is similar to the Fc′ fragments obtained by digestion of γG immunoglobulins with pepsin or papain. These results are in accord with the hypothesis that the immunoglobulin molecule is composed of compact domains, separated by stretches of relatively extended polypeptide chain, and provide a method of isolating these domains for determination of their individual functions.

Analysis of the complete primary structure of the γG1 immunoglobulin[1] Eu by Edelman *et al.* (1969) led them to propose that antibodies consist of a series of compact domains, and that each domain contains at least one active site serving a specific function in the molecule. Direct evidence to support this hypothesis has been obtained from X-ray crystallographic studies (Sarma *et al.*, 1971; Poljak *et al.*, 1972) and electron micrographs (Green *et al.*, 1971; Dourmashkin *et al.*, 1971) of various immunoglobulins. Several studies of limited enzymatic digestion of immunoglobulins have shown that cleavage of the chains between homology regions can be obtained (Porter, 1959; Turner and Bennich, 1968), consistent with the hypothesis that the chains are composed of compact, tightly folded regions linked by more extended stretches of polypeptide chain. Similarly, Bence-Jones proteins and isolated light chains can be cleaved into constant (C_L) and variable (V_L) halves (Solomon and McLaughin, 1969; Karlsson *et al.*, 1969).

We now report the isolation of a new immunoglobulin fragment from subtilisin digests of both the myeloma protein Eu and normal γG immunoglobulin. The fragment corresponds to the C_L and C_H1 regions of the molecule. The V_L, V_H, hinge, and C_H2 regions are extensively degraded. These results are consistent with the domain hypothesis and provide a method for preparing the C_L and C_H1 domains for the detailed study of their functions within the immunoglobulin molecule.

Materials and Methods

Protein Eu was prepared from plasma by ammonium sulfate precipitation and DEAE-cellulose chromatography (Edelman *et al.*, 1968). Fab(t) and Fc(t) fragments of protein Eu were prepared by tryptic digestion as described by Edelman *et al.* (1968), except that the protein was not reduced or alkylated prior to digestion. Eu light chain was isolated from partially reduced and alkylated Eu by the method of Edelman *et al.* (1968). Normal human immunoglobulin (Cohn fraction II, lot C-842) was obtained from Lederle Laboratories, Pearl River, N.Y.

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[1] The nomenclature used for immunoglobulins and their chains and fragments follows that recommended by the World Health Organization (1964). The nomenclature of immunoglobulin homology regions (V_L, C_L, V_H, C_H1, C_H2, and C_H3) is that of Edelman *et al.* (1969).