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PROTEIN CONFORMATIONAL TRANSITIONS IN THE ERYTHROCYTE MEMBRANE

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SUMMARY

Erythrocyte membranes prepared from human red blood cells by hypotonic lysis, contain protein in a mixture of α-helical and "unordered" conformations. Using infrared absorption spectroscopy we have demonstrated that in the presence of adenosine triphosphate and Mg^{2+}, the protein in these membranes undergoes a transition to an anti-parallel β-structure, which is enhanced by the addition of Na^+ and K^+. This transition can be prevented or reversed under conditions which inhibit the adenosine triphosphatase reaction. The results are discussed in the light of data previously obtained by infrared spectroscopy on rat liver mitochondria, in terms of a generalized membrane-energy transduction concept.

INTRODUCTION

In mitochondria, induction of electron transport in the presence of permeant anions effects a morphological expansion of the inner mitochondrial (cristal) membranes, while phosphorylating conditions induce their contraction[1-3]. Several additional "energized and non-energized" states have also been discerned by electron microscopy, each associated with some distinctive functional and permeability characteristics[4,5]. Because such gross changes in membrane morphology and permeability can reasonably be attributed to architectural modulations at the molecular level, numerous investigators have attempted to define these changes by spectrophotometric measurements.

Fluorescent probes such as 1-anilino-8-naphthalene sulphonate, have been widely used to this effect, and do change their fluorescence characteristics in response to energy transitions in mitochondria[6,7] and in submitochondrial particles or mitochondrial membrane fragments[7-11]. The oscillations in fluorescence intensity are slighty but consistently out of phase with the oscillations in respiratory state[6]. Such data, indicating changes in dye binding and/or the polarity of the binding sites are compatible with the occurrence of molecular rearrangements lagging behind the chemical events of electron transport and phosphorylation, but cannot be more precisely interpreted.

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In a different approach, WRIGGLESWORTH AND PACKER[12] measured the optical rotatory dispersion of mitochondria fixed in different respiratory states by the addition of glutaraldehyde. The spectra in respiratory states I or IV were red-shifted and of lower amplitude than those of mitochondria in state II. These data were interpreted solely in terms of the presence of peptide in "unordered" or α-helical conformations in the membranes. This analysis is not justified since WALLACH *et al.*[13] using infrared spectroscopy, have shown that a considerable proportion of the membrane peptide in mitochondrial membranes exists in the anti-parallel β-conformation. Moreover, GRAHAM AND WALLACH[14] have recently shown that the proportion of β-structure increases in the presence of succinate-induced electron transport; becomes predominant during uncoupled electron transport; diminishes during oxidative phosphorylation and is virtually undetectable when electron transport is inhibited.

Quite recently, PENNINSTON AND GREEN[15] have reported that in the simultaneous presence of ATP, Mg^{2+}, Na^+ and K^+, erythrocyte ghosts undergo a process of membrane deformation, which the authors link to the hydrolysis of ATP, and which appears to differ from the types of endovesiculation reported by STECK *et al.*[16].

We have followed up these morphological studies with infrared spectroscopic investigations and find that ATP hydrolysis is indeed associated with alterations in the secondary structure of the membrane proteins. This is the topic of this report.

MATERIALS AND METHODS

Poly-L-lysine (mol. wt. 130000) was obtained from Mann Research Laboratories, New York, N.Y. Aqueous solutions of the polypeptide in "unordered", α-helical and anti-parallel β-structures were prepared by standard methods[17]. Whale skeletal muscle myoglobin was purchased from Seravac Laboratories, Maidenhead, England, and lysozyme from Worthington Biochemicals, Freehold, N.J.

Erythrocyte "ghosts" were prepared from freshly-drawn human blood according to DODGE *et al.*[18] and stored for up to 1 week in 0.25 M sucrose–0.007 M phosphate (pH 7.4) at $-85°$.

Sample preparation for film infrared spectroscopy

Erythrocyte ghosts. After thawing, these were pelleted at 20000 $\times$ g_{av} for 10–15 min (at 4°); washed twice in cold 0.007 M phosphate buffer (pH 7.4) and finally suspended in this medium to a concentration of 2 mg protein per ml. 25 μl of this suspension was layered atop a AgCl disc, 1 mm thick and 24 mm in diameter (Harshaw Chemical Co., Cleveland, Ohio) to form a rectangular (20 mm $\times$ 8 mm) film centred on the disc. A rectangular template was used to insure reproducibility of the films. The layered suspension was frozen, without disturbance of the dimensions of the rectangle, by immersing the disc in liquid nitrogen (—180°): the film was lyophilized and stored *in vacuo*, and in the dark until analyzed.

In studies into the effect of ATP, 5 μl of phosphate buffer containing ATP, Mg^{2+}, Na^+, K^+, EDTA or combinations of these, were added to the membrane suspensions on the disc; mixed in and the whole incubated at room temperature for 2 min prior to freezing. ATP and $MgCl_2$ were added to a final concentration of $2 \cdot 10^{-3}$ M; NaCl and KCl were added to a final concentration of $2.5 \cdot 10^{-2}$ M and $5 \cdot 10^{-3}$ M, respectively, unless indicated otherwise. Matched AgCl discs, of identical cross-

section supporting dried films of 0.007 M phosphate buffer (pH 7.4) (containing appropriate additions) were used in the reference beam.

Polypeptides and proteins. 25–50-μl layers of poly-L-lysine, lysozyme or myoglobin (5–10 mg/ml) were applied to AgCl discs as described above and then dried at room temperature by vacuum desiccation. They were stored *in vacuo* and in the dark when necessary.

Infrared spectroscopy in ^2H_2O.

Erythrocyte "ghosts" washed once in 0.007 M phosphate buffer (pH 7.4) were pelleted (20000 $\times$ g_{av} for 15 min); the buffer meticulously removed and the membranes dispersed in 0.007 M phosphate in ^2H_2O (p^2H 7.4) using a 1-ml disposable syringe with a gauge 18 needle. After 15 min at 0–1°, the membranes were re-sedimented (20000 $\times$ g_{av} for 15 min at 0–1°). The supernatant was discarded and the ^2H_2O wash repeated before final dispersion of the "ghosts" in ^2H_2O–0.007 M phosphate buffer (p^2H 7.4) at a concentration of *c* 3 mg protein per ml. A small volume of this suspension (*c* 0.2 ml) was taken up into a 1-ml syringe and introduced *via* a Luer injection port into an infrared liquid absorption cell, path length 0.5 mm, equipped with IRTRAN 2 windows (Perkin-Elmer Corp., Norwalk, Conn.). An identical matched cell containing ^2H_2O–phosphate buffer was used as the reference. Additions (ATP, Mg^{2+}*, Na^+, K^+ and EDTA in ^2H_2O) were made to both sample and reference cells, to the same final concentrations as described above (except where indicated).

Infrared spectroscopy

All measurements were made with a Perkin–Elmer Model 221 infrared spectrophotometer, using a source intensity of 0.25 A. Spectra were regularly recorded from 1800 to 1600 cm^{-1} at a scan speed of 1/4 wave numbers per sec. Resolution was at all times better than 2 cm^{-1}. Base-lines were periodically checked using either AgCl discs or the infrared liquid absorption cells containing ^2H_2O (to minimize reflections from the 2 windows) in both sample and reference beams. The base lines exhibited a maximum drift of less than 0.02 absorbance unit in the region of interest. Resolution and reproducibility were checked with a standard polystyrene film supplied by the Perkin-Elmer Corp.

ATPase assay

[γ-^{32}P]ATP was prepared according to WEISS *et al.*[19] except that it was deemed unnecessary to concentrate the labelled ATP on Norit. After elution of the nucleotide from the Dowex column, the solution was neutralized with Tris and stored frozen in small samples. The assay reaction was performed in 0.125 ml and contained "ghosts", ATP, Mg^{2+}, Na^+ and K^+ at the concentrations given above. After 30 or 60 min at 37°, liberated ^{32}P was assayed according to the method of WEISS *et al.*[19].

Presentation of data

All infrared spectra have been normalized so that the maximum peak absorption is 1.0 absorbance unit.

* $MgCl_2 \cdot x^2H_2O$ was prepared by placing a known amount of $MgCl_2 \cdot 6H_2O$ in a vacuum desiccator. After 2–3 h the vacuum was released and the dried sample exposed to an atmosphere of ^2H_2O for about 3 h. The deuterated crystals were then suspended in ^2H_2O to the appropriate concentration of $MgCl_2$.

RESULTS

Infrared spectra of "non-energized" erythrocyte membranes

Effect of lyophilization

As shown in Fig. 1, the infrared spectra of membranes, cast from 0.007 M phosphate buffer (pH 7.4) *in vacuo* at 25° or by lyophilization show an essentially identical Amide I band, centred around 1652 cm^{-1}. This confirms data reported by WALLACH AND ZAHLER[20,21] on the plasma membrane of Ehrlich ascites carcinoma cells and of MADDY AND MALCOLM[22,23] and of CHAPMAN *et al.*[24] on erythrocyte "ghosts". The Amide I band of lyophilized erythrocyte "ghosts" does, however, show some variable broadening around 1640 cm^{-1}, in different "ghost" preparations. Although the preparative procedure was carefully controlled it was difficult to avoid some minor variations. In our experience probably the most significant variation was the ease of resuspension of the erythrocyte ghost pellets during haemolysis. According to spectra

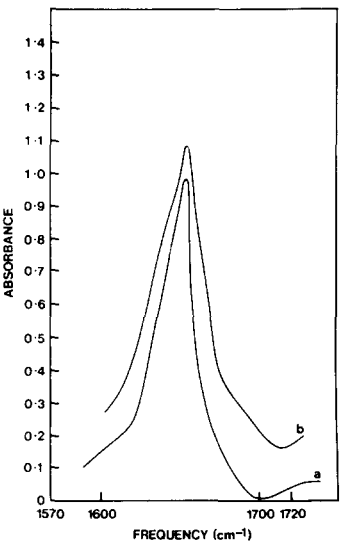

Fig. 1. Amide I band of erythrocyte "ghost" film cast by (a) lyophilization, (b) drying at room temperature. The two curves are displaced by 0.1 absorbance unit.

TABLE I

RECOVERY OF ION-SPECIFIC ATPase IN ERYTHROCYTE "GHOSTS" AFTER LYOPHILIZATION

Erythrocyte ghosts (2.0 mg protein per ml) were shell-frozen in liquid nitrogen and lyophilized. Water was added to the lyophilizate (to the original volume) after exposure of the lyophilizate to an atmosphere at 100% humidity at 4° for 2 h.

	ATPase activity (μmoles ATP hydrolyzed/h per mg protein) in the presence of		*Stimulation by K^+ (%)*
	$Mg^{2+} + Na^+$	$Mg^{2+} + Na^+ + K^+$	
Control erythrocyte "ghosts"	0.410	0.562	37
Lyophilized erythrocyte "ghosts"	0.650	0.862	33

of synthetic polypeptides of known secondary structure, the lack of a significant band at 1625–1640 cm^{-1} indicates little β-structured peptide under these conditions.

Optical activity measurements on Ehrlich ascites cell plasma membrane[25] and erythrocyte membrane[26] suggests a mixture of a α-helix and "unordered" peptide in the membrane protein. This conclusion is supported by, but not amplified by, the infrared spectra, which cannot distinguish between these conformations. Accordingly, helix–coil transitions during film preparation cannot be excluded. However, at the concentrations used (2 mg protein per ml), slow rehydration of the lyophilizate in a water-saturated atmosphere at 4°, prior to bulk resuspension to the original concentration, yields erythrocyte ghosts of biconcave shape, indistinguishable from normal controls and yielding essentially full recovery of (Na^+–K^+)-stimulated ATPase activity (Table I).

Comparison with Amide I spectra of poly-L-lysine and proteins

The film spectra (1600–1800 cm^{-1}) of (a) α-helical poly-L-lysine, (b) myoglobin, (c) 1:1 "unordered": α-helical poly-L-lysine and (d) erythrocyte "ghosts" are given in Fig. 2. In (a) and (b) 250 μg of material was vacuum dried at room temperature to yield rectangular films of standard dimensions. The "ghost" film was deposited by lyophilization and in (c) a disc supporting 125 μg α-helical poly-L-lysine was placed in tandem with a disc bearing 125 μg of the "unordered" polymer in an identically dimensioned and oriented film.

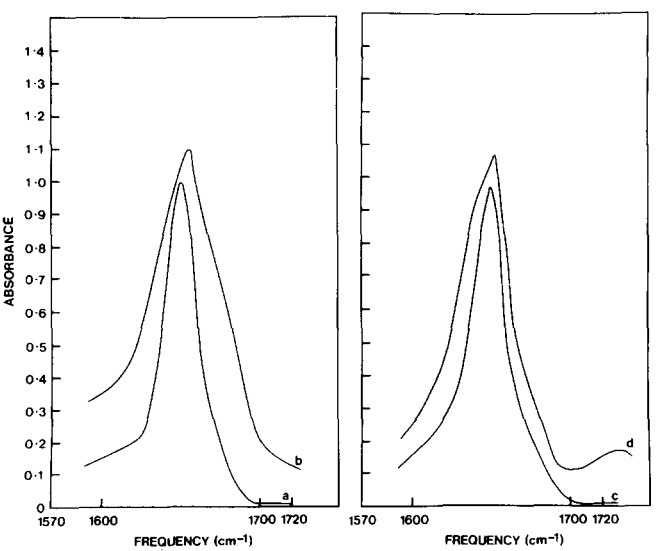

Fig. 2. Amide I band of vacuum-dried films of (a) α-helical poly-L-lysine; (b) myoglobin; (c) 1:1 "unordered": α-helical poly-L-lysine and a lyophilized film of (d) erythrocyte "ghosts". In each panel the two curves are displaced by 0.1 absorbance unit.

The Amide I bands of myoglobin and "ghosts" are appreciably broader than that of α-helical poly-L-lysine. This is not due to the presence of both helical and "unordered" conformations since the artificial conformational mixture (c) yields a spectrum almost identical to that of the purely helical homopolymer (a). The "broadening" observed in (b) and (d) is thus more likely to be due to side chains containing aromatic

residues and/or ionized and unionized carboxyls absorbing at 1600–1650, 1600–1620 and 1670–1710 cm^{-1}, respectively[27].

In an effort to find reference models more suitable than pure synthetic polymers, we have examined discs bearing α-helical poly-L-lysine placed in tandem with discs supporting identically-dimensioned and oriented films of the following: 250 μg mixed amino acids (casein hydrolyzate pH 3.0); 50 μg L-serine (pH 4.0) and a mixture of 50 μg L-phenylalanine and 50 μg ethanolamine. The results, shown in Fig. 3, demonstrate "broadening" in all cases, but with the casein hydrolyzate in particular the carboxylate absorption is clearly excessive. Although we have not yet found a model system contributing sufficient side-chain absorbance without excessive COOH and/or COO^- contributions, our data suggest that the widening of the Amide I band in membranes and proteins is due to side-chain contributions.

Lipid contributions to the Amide I spectra of "ghosts" due primarily to the amide linkage of sphingomyelin, are small[20,21]. In human erythrocytes the mass ratio protein: sphingomyelin is about 6:1 (ref. 28), so that the amide absorption of the lipid cannot be more than 2 % of that of the protein.

From the above it appears that the characteristic absorption of erythrocyte "ghosts" in the Amide I region arises from the peptide linkages and side-chain contributions of membrane proteins.

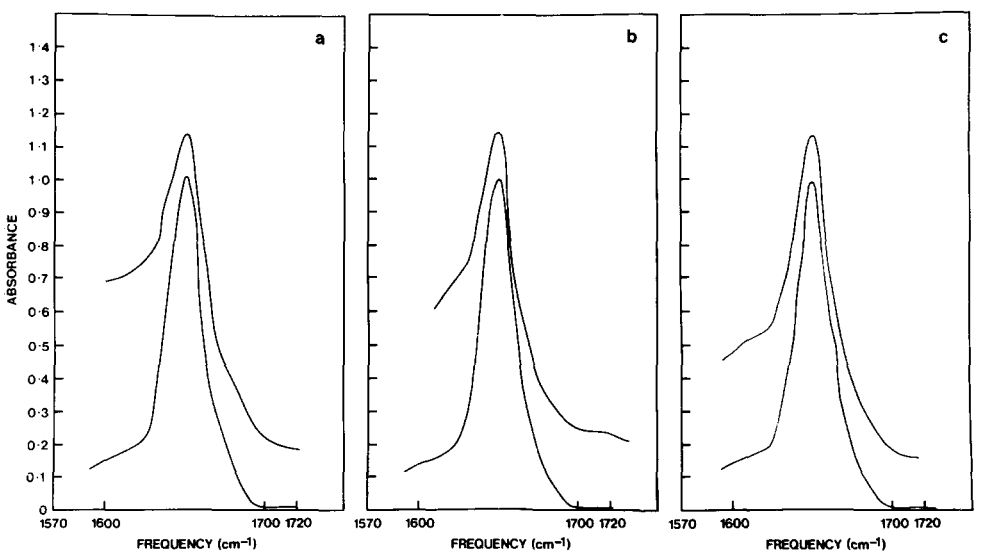

Fig. 3. In each panel the lower spectrum is the Amide I region of 250 μg α-helical poly-L-lysine while the upper spectrum is that of (a) 250 μg α-poly-L-lysine + 250 μg casein hydrolyzate (pH 3.0); (b) 250 μg α-poly-L-lysine + 50 μg L-serine and (c) 250 μg α-poly-L-lysine + 50 μg phenylalanine + 50 μg ethanolamine. The two curves in each panel are displaced by 0.15 absorbance unit.

Amide I spectra of erythrocyte "ghosts" in ^2H_2O

Shortly after transfer of erythrocyte membranes into ^2H_2O-containing 0.007 M phosphate buffer (p^{2}H 7.4) their Amide I spectra (Fig. 4a) resemble those of lyophilized "ghosts". The shift of maximal absorption to 1648–1650 cm^{-1} is also seen with synthetic polypeptides[29]. However, after 2 h in ^2H_2O at 0–1°, or lesser times at higher

temperatures, a shoulder appears at 1635 cm^{-1} (Fig. 4b); this, in the absence of increased absorption near 1690 cm^{-1}. At the same time, the "ghosts" become spherical, losing the biconcave morphology typical in ^2H_2O–0.007 M phosphate buffer (pH 7.4). Also the endocytosis characteristic of "ghosts" in aqueous 0.0007 M phosphate buffer (pH 7.4)[16] is much curtailed when ^2H_2O is substituted for H_2O. The magnitude of the changes in the Amide I band observed upon substitution of ^2H_2O for H_2O depends significantly upon the age of the "ghost" preparation. Freshly prepared "ghosts" show smaller changes under the conditions described than do "ghosts" stored for 3 weeks at −88°. If the "ghosts" are maintained in ^2H_2O at 20° then after 0.5–1 h the absorption of the Amide I band at 1630 cm^{-1} becomes overwhelmingly predominant.

The changes in the form of the Amide I band may arise through deuteration effects *per se*. Free uncharged $-NH_2$ groups exhibit a band between 1600 and 1650 cm^{-1} due to deformation modes. Alterations in the absorption below 1650 cm^{-1} could occur upon deuteration of these free $-NH_2$ groups. The source of these amino groups may be the protein in the membrane itself or some contaminant in the membrane preparation. Variation in the degree of contamination could account for the slight differences in shape of the Amide I band observed with lyophilized preparations. The positive correlation between aging and spectral changes however is difficult to reconcile with the idea of contamination, particularly since considerable washing of the erythrocyte "ghosts" is involved prior to infrared analysis. Furthermore the magnitude of the infrared effect after 0.5–1 h at 20°, and the gross morphological changes which occur in ^2H_2O suggest that significant rearrangements are occurring at the molecular level. The increase in absorption between 1630 and 1640 cm^{-1} may

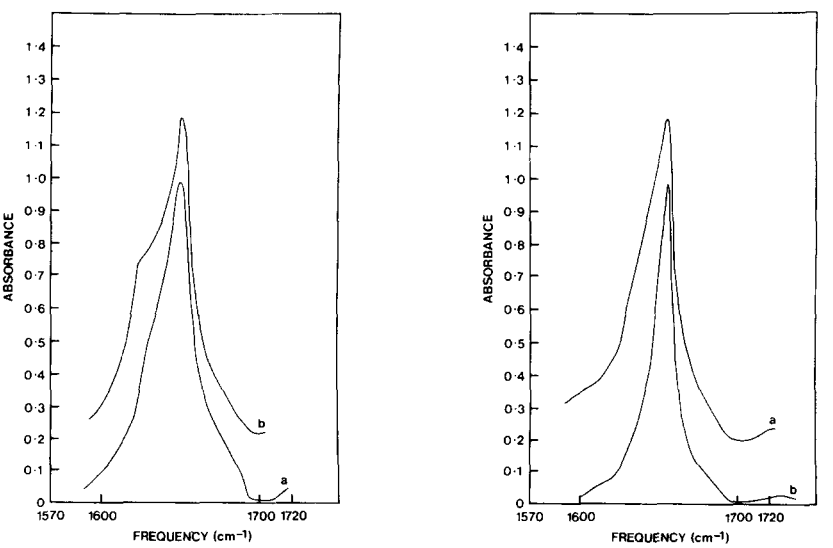

Fig. 4. Amide I band of erythrocyte "ghosts" in ^2H_2O–0.007 M phosphate buffer (p^{2}H 7.4) after (a) 15 min at 0°; (b) 2 h at 0°. The two curves are displaced by 0.2 absorbance unit.

Fig. 5. Amide I band of a film of erythrocyte "ghosts" cast by lyophilization in (a) 0.007 M phosphate buffer (pH 7.4); (b) phosphate buffer containing 0.05 M Na^+, 0.01 M K^+ and 0.002 M Mg^{2+}. The two curves are displaced by 0.2 absorbance unit.

therefore reflect a transition of some of the membrane peptide into a parallel β-structure.

Spectral changes associated with ATP hydrolysis

Lyophilized membranes

In Fig. 5 we show the Amide I spectra of erythrocyte "ghosts" lyophilized from 0.007 M phosphate (pH 7.4), (a) without any additions and (b) upon addition of 0.05 M Na^+ + 0.01 M K^+, + 0.002 M Mg^{2+}: there are no significant differences.

However, simultaneous addition of 0.002 M ATP and 0.002 M Mg^{2+} effects a pronounced increase of absorption at 1630–1640 cm^{-1} together with the appearance of a small shoulder at 1690 cm^{-1} (Fig. 6a). The changes at 1630–1640 cm^{-1} suggest a transition of some of the membrane peptide into a β-conformation. The appearance of the shoulder at 1690 cm^{-1} may further indicate that this β-structure is anti-parallel, although the formation of some strongly hydrogen-bonded carbonyl groups in the membrane phospholipids could also account for this band. There is, however, a good correlation between the magnitude of the changes at 1630–1640 cm^{-1} and those at 1690 cm^{-1}: thus we tentatively consider the 1690 cm^{-1} band to arise from an anti-parallel β-structured peptide. This point will be mentioned again in the DISCUSSION.

The greater the ATPase activity of the membranes, the more β-structure is induced by ATP + Mg^{2+}. Addition of Na^+ and K^+ (50 mM and 10 mM, respectively) stimulates ATP hydrolysis up to 3-fold and effects a proportionally greater change

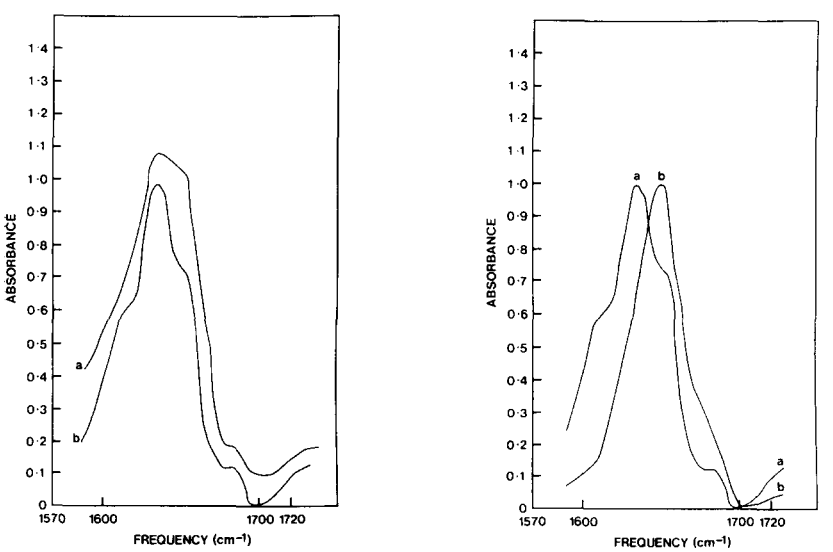

Fig. 6. Amide I band of a film of erythrocyte "ghosts" in 0.007 M phosphate buffer (pH 7.4), cast by lyophilization in the presence of (a) 0.002 M Mg^{2+}–ATP and (b) 0.002 M Mg^{2+}–ATP + 0.05 M Na^+ + 0.01 M K^+. The two curves are displaced by 0.1 absorbance unit. Under identical conditions the ratio of the absorbances at 1655 and 1635 cm^{-1} varied by about 25 % with different membrane preparations depending upon the activity of the ATPase in the preparation.

Fig. 7. Amide I band of a film of erythrocyte "ghosts" in 0.007 M phosphate buffer (pH 7.4), cast by lyophilization in the presence of (a) 0.002 M Mg^{2+}–ATP, 0.05 M Na^+ and 0.01 M K^+; (b) Mg^{2+}, ATP, Na^+ and K^+ as in (a) + 0.002 M EDTA.

TABLE II

EFFECT OF EDTA ON THE ATPase ACTIVITY OF ERYTHROCYTE MEMBRANES

Assay medium	*ATPase activity (μmoles ATP hydrolyzed/h per mg protein)*
ATP	0.23
ATP + EDTA	0
ATP + Na^+ + K^+	0.68
ATP + Na^+ + K^+ + EDTA	0
ATP + Mg^{2+} + Na^+ + K^+	7.6
ATP + Mg^{2+} + Na^+ + K^+ + EDTA	0

towards an anti-parallel β-conformation (Fig. 6b). Abolition of ATP hydrolysis by the addition of EDTA (2 mM) to the ATP–Mg^{2+}-containing medium (± Na^+ and K^+) also eliminated all infrared evidence of a transition into a β-structure (Table II and Fig. 7).

At the ATP concentrations used, and under the conditions of the infrared analysis, the nucleotide does not absorb significantly in the Amide I region. Outside the expected decrease in base line absorbance, the spectrum of a "ghost" film containing ATP was not influenced by placing an ATP film in the reference beam. In addition, the infrared spectrum of a "ghost" film was unaltered by placing an AgCl disc bearing a film of ATP in tandem with the disc supporting the membrane film.

TABLE III

RELATIVE HYDROLYSIS OF ATP, ADP AND ITP BY ERYTHROCYTE GHOSTS

Red blood cell "ghosts" (2 mg protein per ml) in 20 mM Tris (pH 7.4) were incubated with 1 mM nucleotide (ATP, ADP or ITP), 1 mM Mg^{2+}, 20 mM Na^+ and 5 mM K^+ (total vol. 0.3 ml) for 1 h at 37°. Controls containing either no nucleotide or no membrane were similarly incubated. The reaction was terminated by the addition of 0.3 ml 5% trichloroacetic acid. After removal of the precipitate by centrifugation at 1000 × *g* for 10 min (2°); P_i in the supernatant was estimated colorimetrically.

Nucleotide	*Nucleotide hydrolyzed (%)*
ATP	5
ADP	0.5
ITP	2

ITP was hydrolyzed by erythrocyte "ghosts" at about half the rate of ATP and ADP was barely cleaved at all (Table III). Concordantly, the transition to a β-structure was less with ITP than ATP and hardly detectable with ADP (Fig. 8).

Some ATP hydrolysis (and transition towards an anti-parallel β-structure) occurs in the absence of added Mg^{2+} but can be prevented by EDTA; we attribute this to the presence of some bound divalent cation.

Comparison with polypeptide mixtures

To simulate the changes in infrared absorption which we observe with erythrocyte "ghosts" in the presence of ATP and cations, we have recorded the "additive" Amide I spectra of myoglobin films and films of β-structured poly-L-lysine in various tandem combinations. We first established the validity of this approach by determining the absorbance of each substance, using 1, 2, 3 and 4 discs, each supporting identical films cast from 5 μl of a 10 mg/ml solution. As successive discs were placed in the sample beam, a variable attenuator in the reference beam was adjusted to maintain the base line at 1800 cm^{-1} constant. The amplifier gain was adjusted between 4.0 and 4.3 to keep a full scale pen response of 3 sec.

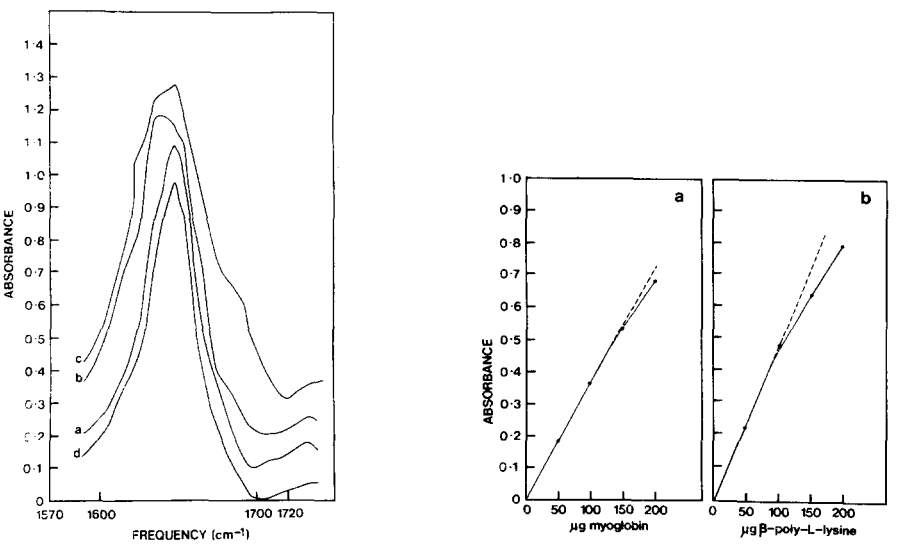

Fig. 8. Amide I band of a film of erythrocyte "ghosts" in 0.007 M phosphate buffer (pH 7.4), cast by lyophilization in the presence of (a) 0.002 M Mg^{2+}; (b) 0.002 M Mg^{2+}–ATP; (c) 0.002 M Mg^{2+}–ITP; (d) 0.002 M Mg^{2+}–ADP. Each curve is displaced from its neighbour by 0.1 absorbance unit.

Fig. 9. (a) Variation in absorbance at 1655 cm^{-1} of air-dried films of myoglobin with "concentration" of myoglobin. (b) Variation in absorbance at 1625 cm^{-1} of air-dried films of β-poly-L-lysine with "concentration" of β-poly-L-lysine.

The results (Fig. 9) indicate an almost linear relationship between absorbance and "concentration" (number of discs). We have accordingly used combinations of discs bearing myoglobin or β-structured poly-L-lysine to generate spectra approximating the following proportions of (α-helix + "unordered"): β-structure = 1:3, 1:2, 1:1, 2:1 and 3:1 (Fig. 10).

It is clear from a comparison of these spectra of mixtures of myoglobin and β-structured poly-L-lysine with those of lyophilized erythrocyte "ghosts" previously incubated with ATP *etc.* that there is a significant "background" absorption in the Amide I region of the latter which is absent in the former. The contribution to the absorption, of protein side chains and light scatter from the lyophilized films is difficult to assess. Moreover band positions and band shapes are difficult to reproduce. Even when in the same conformation, diverse homopolypeptides do not exhibit

precisely identical band positions[29,30], and cannot be assumed to have equivalent band absorbances. Moreover, proteins are usually conformational mixtures, often containing (a) only short segments in any one conformation and (b) sections which are "unordered" but not random, or helical but not precisely α-helical or folded parallel or anti-parallel, but not truly β-structured[31,32]. Under these conditions therefore it is

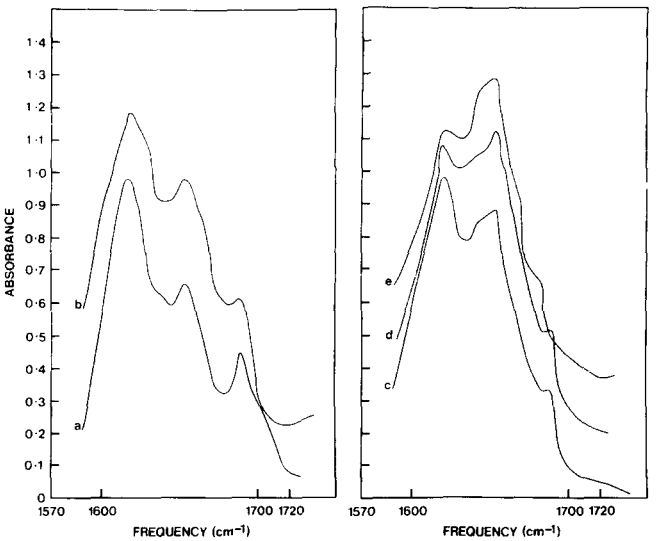

Fig. 10. Amide I bands of air-dried films of mixtures of myoglobin and β-poly-L-lysine in the following ratios: (a) 1:3; (b) 1:2; (c) 1:1; (d) 2:1 and (e) 3:1. In the left-hand panel the two curves are displaced by 0.2 absorbance unit; in the right-hand panel each curve is displaced by 0.15 absorbance unit.

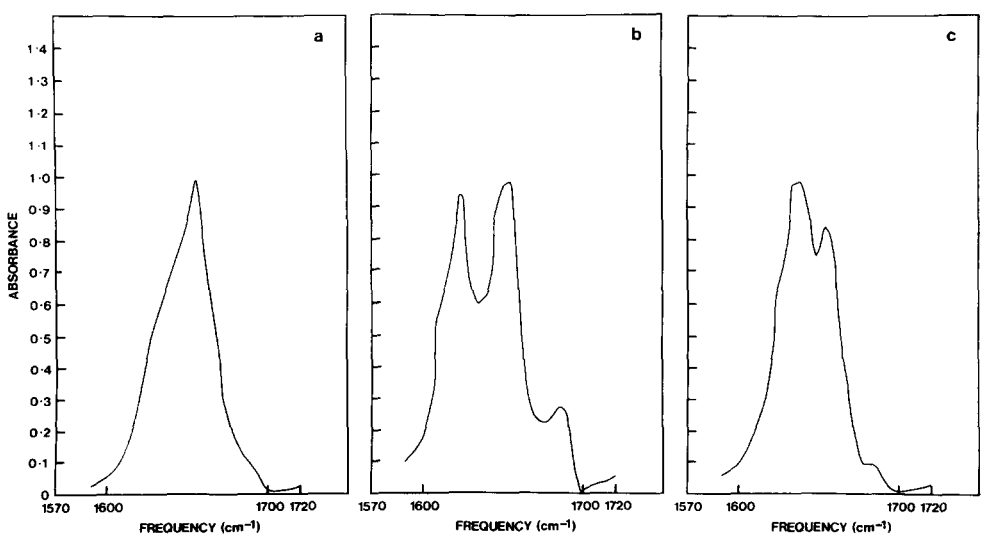

Fig. 11. Amide I band of erythrocyte "ghosts" in ^2H_2O-phosphate buffer (0.007 M, p^{2}H 7.4) containing (a) 0.001 M Mg^{2+}, (b) 0.001 M Mg^{2+}–ATP, (c) 0.001 M Mg^{2+}–ATP + 0.01 M Na^+ + 0.002 M K^+.

considered hazardous to make any firm quantitative assessment of the conformational changes involved.

Effects in ^2H_2O

We have observed an increase in β-structure upon ATP hydrolysis in erythrocyte "ghosts" suspended in ^2H_2O. These experiments were limited to about 15-min incubations, to keep non-specific ^2H_2O effects minimal. Fig. 11a shows the Amide I spectrum of "ghosts" in ^2H_2O–0.007 M phosphate buffer (p^{2}H 7.4) containing 0.001 M Mg^{2+}. Addition of ATP to 0.001 M (Fig. 11b) clearly increases the proportion of anti-parallel β-structure, as indicated by the peak near 1630 cm^{-1} and the shoulder near 1690 cm^{-1}; the latter distinguishes this effect from non-specific ^2H_2O-induced changes. In the presence of Na^+ and K^+ the absorption near 1630 cm^{-1} becomes predominant (Fig. 11c). Addition of EDTA (to 0.001 M) prior to ATP, abolishes the ATP response (Fig. 12a). When added after the ATP, this reverses the spectral changes (Fig. 12b).

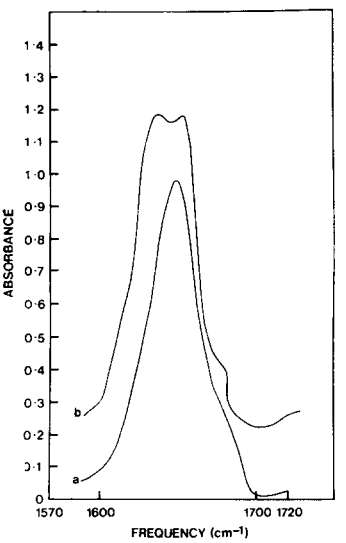

Fig. 12. Amide I band of erythrocyte "ghosts" in ^2H_2O–phosphate buffer (0.007 M, p^{2}H 7.4 containing 0.001 M Mg^{2+}–ATP, 0.01 M Na^+, 0.002 M K^+ and (a) 0.001 M EDTA added prior to the ATP, (b) 0.001 M EDTA added 2 min after the ATP. The curves are displaced by 0.2 absorbance unit.

Because non-specific ^2H_2O effects become predominant after 30–40 min, we could not detect any possible reversal of the ATP-induced changes upon ATP depletion. Also at the low ATP levels needed to yield *c* 25 % hydrolysis in 40 min (50–100 μM) the spectral changes, while significant were less easily measureable than at higher ATP concentrations.

Quantitation of the effects in ^2H_2O poses problems in addition to those mentioned previously. First, the Amide I positions in ^2H_2O differ substantially between diverse polypeptides and between "unordered" and α-helical conformation[29]. Secondly, the ATP-induced effects appear more variable with the ^2H_2O suspension, perhaps because of the tendency of ^2H_2O alone to produce formation of parallel β-structures.

Reproducibility of infrared spectra

All membrane infrared spectra are representative of at least six experiments with different membrane preparations. In duplicate samples in individual experiments, the band positions were reproducible to $\pm$ 1 cm^{-1} and the peak absorbances never varied by more than 6 %. Different membrane preparations under identical conditions exhibited band positions reproducible to $\pm$ 2 cm^{-1}.

Band positions of vacuum-dried films of poly-L-lysine and myoglobin were reproducible to $\pm$ 1 cm^{-1} and the peak absorbances never varied by more than 3 % with triplicate samples.

DISCUSSION

Our measurements document a biologically important conformational lability of erythrocyte membrane proteins: some peptide linkages shift reversibly into a β-structure probably anti-parallel in nature, as an apparent consequence of ATP hydrolysis, and to an extent correlating with the rate of ATP utilization. These circumstances suggest the possibility that some of the chemical energy released by ATP cleavage is "absorbed" *via* this reordering of protein architecture.

We have recorded analogous phenomena in mitochondrial membranes, where the proportion of β-structure relates to the rate of electron transport, being accelerated by DNP, diminished as ATP is produced from added ADP and P_i, and abolished by respiratory inhibitors[14]. These observations lead us to postulate that in membranes the anti-parallel β-structure may serve as the "energy-conserving" conformation often invoked[5,4]. In the erythrocyte membrane this protein conformational alteration may be a prelude to the ion transport initiated by ATP hydrolysis. The problem as to whether the 1690-cm^{-1} peak truly represents the anti-parallel form of a β-structure or whether it is due to some other carbonyl stretching should be resolved by further experimentation. Infrared analysis following lipid extraction of a lyophilized film of erythrocyte ghosts previously exposed to ATP–Mg^{2+} will determine the relative contribution of lipid carbonyl groups to this absorption at 1690 cm^{-1}.

The appearance of β-conformation in membranes under certain conditions gives no special clue as to their overall molecular organization, since this secondary structure is also present in soluble globular proteins such as lysozyme[33], carboxypeptidase[31], α-chymotrypsin,[34,35] and trypsin[35].

We have pointed out the difficulties in quantifying the proportion of β-conformation, and any figures quoted for the percentage of β-structure in the "energized" membranes must be regarded as approximate, until more definitive experiments have been carried out with synthetic polypeptides containing segments of different secondary structures. We need not invoke, however, an extensive refolding of peptide chains to account for the spectral changes observed. Thus, both α-chymotrypsin[34] and carboxypeptidase[31] contain anti-parallel loops in which only a fraction of the peptide linkages are orientated so as to form β-type hydrogen-bonding. Such regions are deemed "unordered", using infrared spectroscopic criteria, but small rotations about the C–N–C linkages would juxtapose the loops so as to increase the amount of hydrogen bonding. In principle, therefore, small structural rearrangements could convert many peptide segments from a spectroscopically "unordered" array to an anti-parallel β-structure.

The fact that substitution of H_2O by ^2H_2O also induces β-structuring in erythrocyte "ghost" peptides is compatible with the postulated conformational lability of membrane proteins. It is also not suprising, since protein structures are stabilized by entropic effects and hydrogen bonding, both of which are altered by replacement of H_2O by ^2H_2O (ref. 36–39). Whatever the origin of the effects observed in erythrocyte "ghosts", deuteration can clearly effect some departures from the native structure. This may involve merely local irregularities in "ordered" protein structure or the establishment of "deuterium bonding" where hydrogen bonding is sterically unfavourable.

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