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Target sizes of human erythrocyte membrane Ca^{2+}-ATPase and Mg^{2+}-ATPase activities in the presence and absence of calmodulin

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We have investigated the subunit structure of Ca^{2+}-transport ATPase in human erythrocyte membranes using radiation inactivation analysis. All inactivation data were linear on a semilog plot down to at least 20% of the control activity. We found a target size for the calmodulin-dependent Ca^{2+}-ATPase activity of 331 kDa, consistent with the presence of this enzyme as a dimer in calmodulin-depleted ghosts. Membranes which had been saturated with calmodulin before irradiation yielded a a similar size of 317 kDa, implying that activation of Ca^{2+}-transport ATPase by calmodulin does not involve significant change in oligomeric structure. Basal (calmodulin-independent) Ca^{2+}-ATPase activity corresponded to a size of 290 kDa, suggesting that this activity resides in the same, or similar-sized, complex as the calmodulin-dependent activity. Mg^{2+}-ATPase activity, however, was found to reside in a smaller complex of 224 kDa, which proved to be statistically distinct from the target size of Ca^{2+}-ATPase activity. It would appear that Mg^{2+}-ATPase is a distinct entity whose function is likely unrelated to the Ca^{2+}-transport ATPase.

Introduction

The Ca^{2+}-transport ATPase of human erythrocyte membrane is a prototype for the calmodulin-regulated calcium pumps found in the plasma membrane of all eukaryotic cells since it has been extensively characterized with respect to its structural features, reaction mechanism, and regulation by calmodulin [1]. Essential to the understanding of the structure and function of this fundamental integral membrane protein is knowledge of its subunit structure in the membrane, and whether this structure is influenced by binding of the regulatory protein calmodulin.

A simple and direct approach to this problem is the technique of radiation inactivation analysis. For the application of this method, samples are irradiated with increasing doses of radiation, typically high-energy electrons or gamma rays, and the molecular size of the corresponding activity is calculated from the dose dependency of its inactivation [2]. Two groups have recently applied this method to the human erythrocyte Ca^{2+}-transport ATPase [3,4]. Although both studies indicated that the calmodulin-independent activity probably corresponds to a dimer of the enzyme, the bi-exponential inactivation curves of Cavieres [3] for calmodulin-dependent Ca^{2+}-ATPase activity suggested that, in addition to the dimeric form, some of this activity corresponds to larger structures of up to 1 000 000 molecular weight. Minocherhomjee et al. [4] did not report on the target size of

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Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetate.

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calmodulin-dependent Ca^{2+}-ATPase activity. In addition, neither group investigated the interesting possibility that binding of calmodulin to Ca^{2+}-transport ATPase before irradiation might show a change in target size which could be related to the regulatory function of calmodulin.

In order to investigate this matter further, we have measured the target size of Ca^{2+}-transport ATPase using carefully controlled methods (10 MeV electrons and frozen, not lyophillized, samples) calibrated against standards of known molecular size. In addition, we have investigated for comparison the target sizes of basal (calmodulin-independent) Ca^{2+}-ATPase and Mg^{2+}-ATPase activities, as well as the effect of calmodulin binding on the target sizes of Ca^{2+}-ATPase and Mg^{2+}-ATPase activities. A preliminary report of this work has appeared [5].

Materials and Methods

Preparation of calmodulin-deficient human red blood cell membranes

One unit of blood (O^+) was obtained from a local blood bank within 5 days of donation. Membranes were prepared by 1:15 lysis in ice-cold buffer (10 mM imidazole/HCl (pH 7.6) and 0.1 mM EDTA) of saline-washed red blood cells (Gietzen, K., Dick, B., Hauser, U. and Bader, H., manuscript in preparation). The pellet obtained by centrifugation at $12000 \times g$ for 20 min was then washed twice in the same buffer, and a third time in buffer without EDTA. The final pellet containing leaky ghosts was resuspended in 0.5 M sucrose, 0.1 M KCl, 0.5 mM $MgCl_2$, 5 mM Mops buffer (pH 7.0), and 10 mM dithiothreitol, diluted to 2 mg protein/ml, and frozen in liquid nitrogen in 1 ml aliquots for irradiation. In each experiment, a parallel sample was saturated with calmodulin by supplementing the usual membrane suspension with 40 μg calmodulin/ml and 40 μM $CaCl_2$, and allowing the mixture to stand for 10 min at room temperature before freezing. Under these conditions the Ca^{2+}-transport ATPase becomes saturated with calmodulin [6].

Sample irradiation

Samples were shipped to Roskilde at liquid nitrogen temperature, irradiated under nitrogen using a linear accelerator producing electrons at 10 MeV [7], and returned to Ulm at liquid nitrogen temperature for analysis. Doses were measured by thermocalorimetry [8]. Temperature was controlled before each pass through the beam by laying the samples on a salted ice bed adjusted to $-15°C$. Heat generated by irradiation warmed the ice to a maximum of $-5°C$, so the irradiation temperature was considered to be $-10 \pm 5°C$. The samples remained frozen throughout the procedure.

Enzyme assays

ATPase activities were determined at 37°C by measuring release of inorganic phosphate [9]. The assay medium contained 0.1 M KCl, 25 mM Mops (pH 7.0), 0.25 mM ouabain, 1 mM MgATP, 2 mM $MgCl_2$, and 36 μM free Ca^{2+} established by a CaEGTA/MgATP buffer system [10]. For Mg^{2+}-ATPase activity 0.4 mM MgEDTA was substituted for $CaCl_2$, and the protein concentration was 250 μg/ml. For Ca^{2+}-ATPase activity the protein concentration was 125 μg/ml (basal) or 50 μg/ml + 50 nM calmodulin. The samples were preincubated in assay medium for 10 min at 37°C, then further preincubated with or without calmodulin for 10 min at 37°C. The reaction in 0.5 ml was carried out for 20 min at 37°C and stopped by addition of 2 ml of an ice cold solution containing 5.4% (v/v) perchloric acid, 0.51% (w/v) ammonium molybdate, 0.027% (w/v) malachite green, and 0.04% (v/v) Triton X-405 [9]. After addition of 0.5 ml of 34% (w/v) sodium citrate, the color was developed for exactly 30 min at 25°C, and the absorption at 660 nm was measured. Each sample was measured in triplicate.

Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (obtained from Sigma Chemical Co., Munich) was added as standard to the samples before freezing in preparation for irradiation at 5 units/ml. After analysis of ATPase activities, the glucose-6-phosphate dehydrogenase activity of the samples was determined [11] along with the endogenous acetylcholinesterase activity [12].

Analysis of the data

Inactivation data were represented in a semilog plot and analyzed by linear regression analysis. Target sizes were calculated using the formula of

Kepner and Macey [2], $M_r = 6.4 \cdot 10^{11}/D_{37}$, where M_r is the molecular size in daltons and D_{37} is the radiation dose in rads which yields 37% of the control (non-irradiated) activity. Correlation coefficients for inactivation curves with 8–10 points were typically 0.99 or better. Since the standard enzymes gave approximately the expected values, no further correction of the target sizes was made. An exception was one experiment where a correction factor of 1.7 was required based on the standards.

Results

We measured the inactivation of three activities in human erythrocyte membrane as a function of increasing radiation dose: Calmodulin-dependent Ca^{2+}-ATPase, calmodulin-independent or basal Ca^{2+}-ATPase, and Mg^{2+}-ATPase. All three gave straight lines on a semilog plot down to at least 20% of the control activity. The results have been presented in two ways: (1) In the figures all experiments have been combined and the variations in radiation dose for each point (less than $\pm 10\%$) have been ignored for the sake of graphical representation. (2) Each experiment has been handled separately using the measured irradiation dose for each point, and the resulting molecular sizes averaged together (Tables I and II).

The inactivation of calmodulin-dependent Ca^{2+}-ATPase activity from samples which had been irradiated in the presence and absence of calmodulin is depicted in Fig. 1. The D_{37} values obtained were 1.97 Mrad in the absence of calmodulin and 2.06 Mrad in the presence of calmodulin, corresponding to target sizes of 325 and 311 kDa, respectively. Similar plots were obtained using lyophilized samples ($M_r = 261\,000$ irradiated in the absence of calmodulin, data not shown). Inactivation curves for basal Ca^{2+}-ATPase activity are shown in Fig. 2 ($D_{37} = 2.14$, $M_r = 299\,000$) and for Mg^{2+}-ATPase activity in Fig. 3 ($D_{37} = 3.09$, $M_r = 207\,000$), both measured in the absence of calmodulin.

In Table I the results of all experiments are presented, where each experiment has been handled as a separate entity. The results are comparable with those presented in Figs. 1–3.

We found in general that the activities of non-irradiated controls were fairly stable to freeze-thawing and shipment, especially when the samples had been saturated with calmodulin. This is

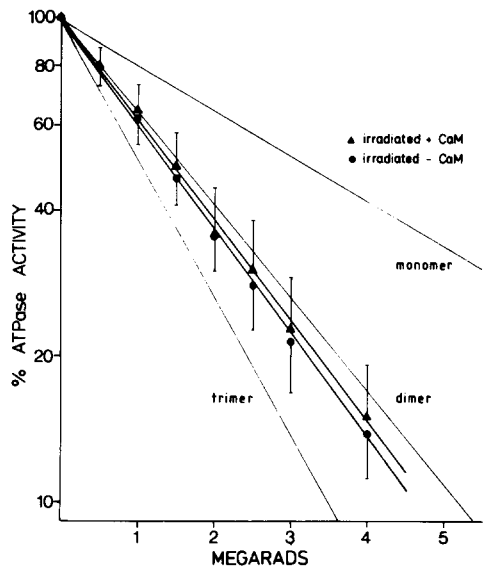

Fig. 1. Radiation inactivation of calmodulin-dependent Ca^{2+}-ATPase activity. Calmodulin-dependent Ca^{2+}-ATPase activity is depicted as a function of radiation dose for erythrocyte membranes irradiated in the presence (▲) and absence (●) of calmodulin. The results of seven experiments are shown with standard deviations. The target sizes calculated for these curves were 311 (▲) and 325 (●) kDa. The theoretical values which would be expected for the monomer (140000 molecular weight), dimer, and trimer, as computed using the equation of Kepner and Macey [2], are also indicated. $100\% = 64.2 \pm 9.3$ (S.D.) nmol/min per mg protein for samples shipped in the presence of calmodulin and 58.4 ± 10.2 nmol/min per mg protein for samples shipped in the absence of calmodulin.

TABLE I

TARGET SIZES OF Ca^{2+}-ATPase AND Mg^{2+}-ATPase ACTIVITIES OF HUMAN ERYTHROCYTE MEMBRANES

CaM, calmodulin. The target size is presented as mean ± S.E.; number of experiments in parentheses.

Activity	Target size (kDa)
Irradiated – CaM	
CaM-dependent Ca^{2+}-ATPase	$331 \pm 13(7)$
Basal Ca^{2+}-ATPase	$290 \pm 38(4)$
Mg^{2+}-ATPase	$224 \pm 22(4)$
Irradiated + CaM	
CaM-dependent Ca^{2+}-ATPase	$317 \pm 20(9)$
Mg^{2+}-ATPase	$254 \pm 16(4)$

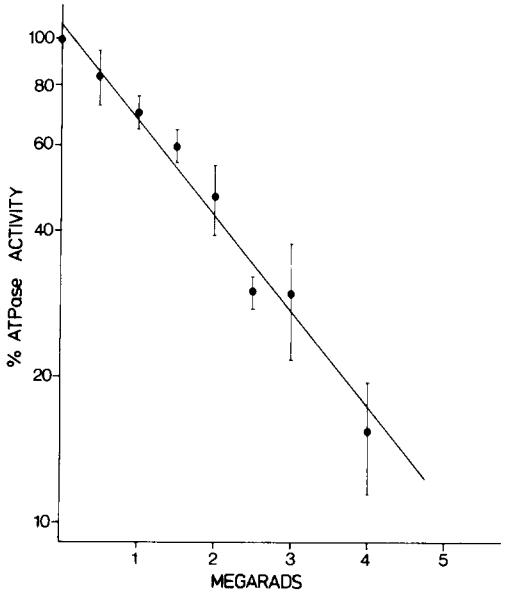

Fig. 2. Radiation inactivation of basal Ca^{2+}-ATPase activity. The decline in calmodulin-independent (basal) Ca^{2+}-ATPase activity is plotted versus the radiation dose. Samples were irradiated in the absence of calmodulin. Results of four experiments are shown with standard deviations. The molecular size corresponding to this activity was calculated to be 299 kDa. Mg^{2+}-ATPase activity has been subtracted. 100% = 14.0 ± 3.1 (S.D.) nmol/min per mg protein.

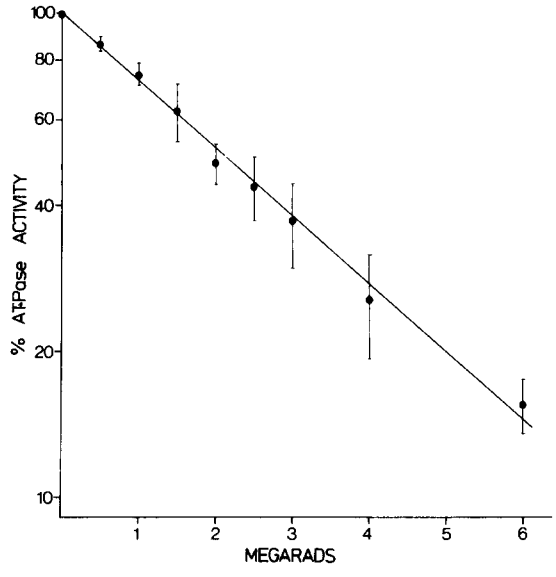

Fig. 3. Radiation inactivation of Mg^{2+}-ATPase activity. Inactivation of Mg^{2+}-ATPase activity is shown for four experiments with standard deviations. Samples were irradiated in the absence of calmodulin; similar results were obtained for samples irradiated in the presence of calmodulin. Calculated target size was 207 kDa. 100% = 5.9 ± 1.9 (S.D.) nmol/min per mg protein.

consistent with the previous observation that addition of calmodulin stabilizes purified Ca^{2+}-transport ATPase [15]. Samples shipped without calmodulin present occasionally lost up to 30% of their calmodulin-dependent Ca^{2+}-ATPase activity, whereas basal Ca^{2+}-ATPase and Mg^{2+}-ATPase activities were more stable.

Finally, we measured simultaneously the target sizes of two control enzymes, one endogenous (acetylcholinesterase) and one exogenous (glucose-6-phosphate dehydrogenase). The results are presented in Table II. In both cases the results obtained were reasonably close to the values previously obtained using other methods. We chose the monomer molecular weight for acetylcholinesterase (80000, Ref. 13) since the presence of 10 mM dithiothreitol in our medium would have reduced the disulfide-linked dimer normally found in human erythrocyte membranes [16].

TABLE II

TARGET SIZES OF ENZYME STANDARDS

The target size is presented as mean ± S.E.; number of experiments in parentheses.

Standard	Molecular weight	Target size (kDa)
Acetylcholinesterase	80000 (Ref. 13)	71.2 ± 2.6(6)
Glucose-6-phosphate dehydrogenase	103700 (Ref. 14)	133.3 ± 3.6(7)

Discussion

The monomer molecular weight of Ca^{2+}-transport ATPase from human erythrocyte membrane has been measured by SDS-polyacrylamide gel electrophoresis of the purified enzyme to be 130000–150000 [10,17]. We find the target size for the calmodulin-dependent activity of this enzyme to be approximately 320000–330000 (Table I), which is consistent with the dimeric form. Since our inactivation curves are linear on a semilog plot down to at least 20% of the control actiivty, we conclude that at least 80% of the Ca^{2+}-transport ATPase probably exists as a dimer in the mem-

brane. We extended this finding with the observation that the target size does not change in the presence of calmodulin. It is now clear that activation of erythrocyte Ca^{2+}-transport ATPase by calmodulin does not involve gross structural changes at the quaternary level.

We were not able to reproduce the biphasic inactivation curves found by Cavieres [3] for calmodulin-dependent Ca^{2+}-ATPase activity. Since lyophilized samples had been used in that study, we considered the possibility that the process of lyophilization might induce aggregation of proteins in the sample. However, we obtained single exponential inactivation curves even with lyophilized samples, yielding a target size similar to that obtained with frozen samples. It should be noted that any form of secondary inactivation (i.e., any unaccounted factor other than radiation) which reduces the activity of the samples relative to the control would give rise to biexponential inactivation curves.

Our target size for the Mg^{2+}-ATPase of erythrocyte membrane (224 000) is somewhat lower that the previously reported values from Kepner and Macey (250 000, Ref. 2) and Minocherhonjee et al. (290 000, Ref. 4). In contrast to the results of Minocherhomjee et al., we found that the target size of Mg^{2+}-ATPase was smaller than that of Ca^{2+}-ATPase. The difference was shown to be significant using Student's t-test ($p < 0.01$). This finding would indicate that erythrocyte Mg^{2+}-ATPase activity corresponds to an enzyme other than the Ca^{2+}-ATPase. This is reasonable, considering that Mg^{2+}-ATPase activity does not copurify with Ca^{2+}-transport ATPase by calmodulin affinity chromatography [10]. The target size of Mg^{2+}-ATPase would be consistent with a dimer of 100 000 to 120 000 molecular weight subunits, analogous to the calmodulin-independent Ca^{2+}-ATPases of sarcoplasmic reticulum [18,19]. Since the function of Mg^{2+}-ATPase activity is unknown, it is interesting to speculate whether it might correspond to a Mg^{2+} pump in the erythrocyte membrane. Considering that the physiological intracellular Mg^{2+} concentration is lower than expected from equilibrium conditions [20], there is reason to suspect that an outwardly-directed Mg^{2+} pump might exist in the plasma membrane of most cells.

The calmodulin-independent or basal Ca^{2+}-ATPase, on the other hand, gave a target size similar to the calmodulin-dependent fraction (Table I). No statistically significant difference was observed between basal and calmodulin-stimulated Ca^{2+}-ATPase activities using Student's t-test ($p > 0.2$). We find it therefore unlikely that basal Ca^{2+}-ATPase activity corresponds to a different form of the enzyme, although small differences in size would not be detected by this method.

With respect to the binding of calmodulin the question arises, why isn't the presence of bound calmodulin detectable as a larger target size for Ca^{2+}-transport ATPase irradiated in the presence of calmodulin? Assuming a 1 : 1 binding stoichiometry, calmodulin could increase the observed target size by only 34 000, or about 10% the size of a dimer. This amount approximates the error in the measurement of the target size and therefore would not be detected.

In our study we have not used a temperature correction factor [21] because our standards gave target sizes reasonably close to the expected values, one 11% low, the other 28% high (Table II). Moreover, we used a buffer containing sucrose, which has been found to yield larger-than-expected target sizes (Nielsen, M., unpublished studies). This may have compensated for the 20% decrease in target size normally found at $-10°C$ compared with room temperature.

The method of radiation inactivation analysis is ideally suited for measuring the size of protein oligomeric complexes in situ without the need for disruptive isolation techniques. However, it must be noted that this method is sensitive to the minimal structural unit which allows effective transmission of the absorbed energy, and not necessarily to the minimal functional unit, as sometimes stated [22]. This has been shown by at least four studies [18,19,23,24] in which the destruction of the polypeptide of interest was followed by electrophoresis. It is clear that when the polypeptide chains of a complex are broken at a rate corresponding to a given oligomeric size, the complex itself is being physically destroyed, and not only its function. Under such conditions, conclusions about the minimal functional unit must be drawn with care, since the functional unit could well reside in only a portion of the target. Furthermore, it is often the case that protein complexes

give a target size smaller than the known complete oligomeric complex [2], presumably because the energy generated by a 'hit' is not always transferred between subunits. The target size of an activity thus represents a minimal estimate of the structural unit in which the activity resides. Moreover, in impure systems the possibility of heterogeneous complexes cannot be ruled out. Unfortunately, purification of membrane-bound enzymes with detergents often destroys the native subunit structure, making extrapolation from purified or reconstituted systems to native systems at best risky.

This study is the first of its kind in seeking an explanation of calmodulin-induced enzyme activation at the level of quaternary structure. Only one other calmodulin-dependent enzyme has been investigated using radiation inactivation, namely the brain cyclic nucleotide phosphodiesterase [25]. In that study, however, no attempt was made to detect possible changes in subunit structure induced by calmodulin. Nevertheless, physiologically relevant changes in quaternary organization have been observed using radiation inactivation, and shown to play an important role in the function regulation. An example is the interaction between adenyl cyclase and glucagon receptor in liver [26]. The utility of radiation inactivation analysis in elucidating quaternary interactions has further been demonstrated in studies of the regulation of brain chloride channels by γ-aminobutyric acid and benzodiazepine receptor ligands (Nielsen, M., Honori, T. and Braestrup, C., manuscript in preparation). These studies have revealed the apparent coexistence of chloride channels, γ-aminobutyric acid receptors, and benzodiazepine receptors in an allosterically- and structurally-linked tetrameric complex of approximately 500 kDa.

It is interesting to note that all ion transport systems which have been investigated to date seem to consist of dimers or higher order oligomers in the membrane [25,18,19,27,28]. It seems likely that multisubunit complexes are the most efficient means toward forming and regulating a hydrophilic channel in the hydrophobic lipid environment of the membrane, in keeping with the alternating access concept of membrane transport [29,30].

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References

1 Schatzmann, H.J. (1982) in Membrane Transport of Calcium (Carafoli, E., ed.), pp. 42–108, Academic Press, New York
2 Kepner, G.R. and Macey, R.I. (1968) Biochim. Biophys. Acta 163, 188–203
3 Cavieres, J.D. (1984) Biochim. Biophys. Acta 771, 241–244
4 Minocherhomjee, A.M., Beauregard, G., Potier, M. and Roufogalis, B.D. (1983) Biochem. Biophys. Res. Commun. 116, 895–900
5 Hymel, L., Nielsen, M. and Gietzen, K. (1984) Cell Calcium 5, 287(A9)
6 Gietzen, K., Wuthrich, A. and Bader, H. (1982) Mol. Pharmacol. 22, 413–420
7 Nielsen, M., Klimek, V. and Hyttel, J. (1984) Life Sci. 35, 325–332
8 Fielden, E.M. and Holm, N.W. (1970) Manual on Radiation Dosimetry (Holm, H.W. and Berry, R.J., eds.), pp. 261–309, Marcel Dekker, Inc., New York
9 Lanzetta, P.A., Alvarez, L.J., Reinach, P.S. and Candia, O.A. (1979) Anal. Biochem. 100, 95–97
10 Gietzen, K., Tejcka, M. and Wolf, U. (1980) Biochem. J. 189, 81–88
11 Decker, L.A. (ed.) (1977) Worthington Enzyme Manual, Worthington Biochemical Corp. Freehold, NJ
12 Steck, T.L. and Kant, J.A. (1972) Methods Enzymol. 31, 172–180
13 Ott, P., Jenny, B. and Brodbeck, U. (1975) Eur. J. Biochem. 57, 469–480
14 Olive, C. and Levy, H.R. (1971) J. Biol. Chem. 246, 2043–2046
15 Gietzen, K. and Kolandt, J. (1982) Biochem. J. 207, 155–159
16 Ott, P., Ariano, B.H., Binggeli, Y. and Brodbeck, U. (1983) Biochim. Biophys. Acta 729, 193–199
17 Niggli, V., Penniston, J.T. and Carafoli, E. (1979) J. Biol. Chem. 254, 9955–9958
18 Hymel, L., Maurer, A., Berenski, C., Jung, C.Y. and Fleischer, S. (1984) J. Biol. Chem. 259, 4890–4895
19 Chamberlain, B.K., Berenski, C., Jung, C.Y. and Fleischer, S. (1983) J. Biol. Chem. 258, 11997–12001
20 Weingart, R. (1985) in Intracellular Calcium Regulation (Bader, H., Gietzen, K., Rosenthal, J., Rudel, R. and Wolf, H.U., eds.), Manchester University press, Manchester, in the press
21 Kempner, E. and Haigler, H.T. (1982) J. Biol. Chem. 257, 13297–13299
22 Kempner, E.S. and Schlegel, W. (1979) Anal. Biochem. 92, 2–10
23 McIntyre, J.O., Churchill, P., Maurer, A., Berenski, C.J.,

Jung, C.Y. and Fleischer, S. (1983) J. Biol. Chem. 258, 953–959
24 Saccomani, G., Sachs, G., Cuppoletti, J. and Jung, C.Y. (1981) J. Biol. Chem. 256, 7727–7729
25 Kincaid, R.L., Kempner, E., Manganiello, V.C., Osborne, J.C., Jr. and Vaughan, M. (1981) J. Biol. Chem. 256, 11351–11355
26 Schlegel, W., Kempner, E.S. and Rodbell, M. (1979) J. Biol. Chem. 254, 5168–5176
27 Ellory, J.C., Green, J.R., Jervis, S.M. and Young, J.D. (1979) Proc. Physiol. Soc. 295, 10P–11P
28 Schrijen, J.J., Van Groninger-Luyben, W.A.H.M., Nauta, H., De Pont, J.J.H.H.M. and Bonting, S.L. (1983) Biochim. Biophys. Acta 731, 329–337
29 Singer, S.J. (1974) Annu. Rev. Biochem. 43, 805–833
30 Tanford, C. (1983) Annu. Rev. Biochem. 52, 379–409