7

STOPPED-FLOW FLUORIMETRY USING VOLTAGE-SENSITIVE FLUORESCENT MEMBRANE PROBES

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7.1 INTRODUCTION

Conceptually, stopped flow is probably the simplest of all rapid-reaction techniques. Its use is not specific to pumps, channels, and transporters, but in combination with voltage-sensitive fluorescent membrane probes, it has yielded much valuable information on their kinetics and mechanisms. Stopped-flow involves the rapid mixing of two reactant solutions, each simultaneously delivered into an observation chamber after the flow of the reactants has ceased. The detection of the course of the reaction of interest can be via UV/visible spectrophotometry or conductometry, but in this chapter, we will concentrate on fluorometric detection. The time resolution that can be achieved is determined by the time required to mix the two reactants, which, using specially designed mixing jets, can be as short as 1–2 ms. There is a huge range of biochemical and chemical reactions that occur over the millisecond-to-second timescale. Therefore, apart from being the most conceptually simple, rapid-reaction technique, stopped-flow is also the most versatile and most widely used. In fact, in a recent review, Olsen and Gutfreund [1] expressed the opinion that stopped-flow, together with the related quenched-flow technique, has probably resulted in more

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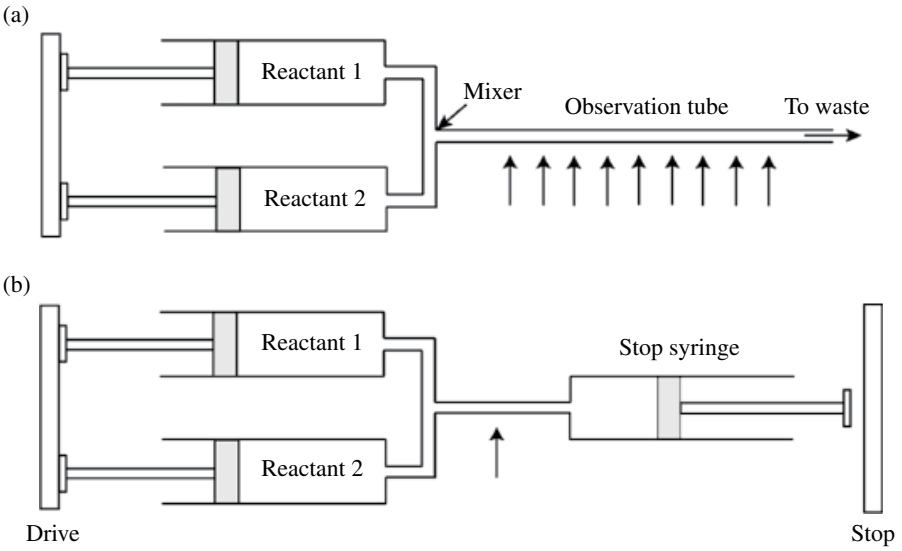

FIGURE 7.1 Basic design of the flow cells of the continuous-flow (a) and stopped-flow (b) techniques. Adapted from Ref. 2 by permission of Oxford University Press.

major contributions to enzymology, molecular biology, and many areas of chemistry than some developments that have been rewarded with the Nobel Prize.

The history of the stopped-flow technique is that it evolved out of economic necessity from a previously developed technique: continuous-flow. The continuous-flow technique involves mixing two reactants, which then flow with a constant velocity down a long tube. If the flow velocity is constant and accurately known, then each distance down the tube past the mixing point corresponds to a particular reaction time (see Fig. 7.1a). By making measurements of the extent of reaction at different distances along the tube, a complete reaction profile can be constructed. This method was first developed by Rutherford [3] in 1897 while working with J. J. Thomson at the Cavendish Laboratory in Cambridge for measuring the rate of recombination of gaseous ions produced by exposure to X-ray radiation. The same concept was taken up in the 1920s by Hartridge and Roughton [4] from the Physiological Laboratory, Cambridge, for the use on solution-phase reactions, in particular the reactions of ligands with hemoglobin and myoglobin. Although excellent time resolution can be achieved using the continuous-flow technique, its major drawback in studying biochemical reactions is that flow must be maintained for considerable lengths of time and hence large amounts of reactants are required. This may not have been a major issue for Hartridge and Roughton if they had sufficient PhD students willing to offer their blood for hemoglobin purification or a nearby abattoir, but for research on more precious proteins, it could well be prohibitive. To reduce the amount of material consumed, in 1936, Millikan [5], a collaborator of Hartridge and Roughton's, devised a miniaturized continuous-flow apparatus, but for many biochemical reactions, the expenditure of biological reactants was still too great and the technique has never become widely used.

To reduce the amount of material required further, Britton Chance [6–10], working in 1940 for a time at Millikan and Roughton's laboratory in Cambridge before returning to the University of Pennsylvania, proposed and developed the stopped-flow technique. In this technique, after a continuous-flow period, the flow is abruptly stopped and the course of the reaction is monitored within the observation chamber. Although the time resolution achievable is not as good as in the continuous-flow method, stopped-flow significantly reduces the expenditure of expensive chemicals or biochemicals. It also allows the complete time course of a reaction to be followed in a single measurement, rather than having to take a series of measurements at different positions as is the case with continuous-flow. Stopped-flow has since become a hugely successful technique. Once commercial instruments became available in the 1960s, the use of stopped-flow was no longer restricted to a small number of specialized laboratories. Nowadays, stopped-flow instruments are in most well-equipped biochemistry or chemistry departments around the world.

7.2 BASICS OF THE STOPPED-FLOW TECHNIQUE

7.2.1 Flow Cell Design

The method has undergone many technical improvements since Britton Chance's first stopped-flow instruments. However, the most fundamental has probably been the introduction in the mid-1950s of the stopping syringe by Quentin Gibson [11–13], then at the University of Sheffield, but who collaborated extensively on the mechanisms of globin proteins with Roughton, the pioneer of continuous-flow. The most important aspect of any flow method is complete mixing, which is only possible if the flow is turbulent, not laminar. Gibson realized this necessitated very abrupt stopping for the stopped-flow method. To achieve this, he introduced a stopping syringe at the outlet from the observation chamber (see Fig. 7.1b). During the continuous-flow period of a stopped-flow measurement, Gibson's stopping syringe gradually fills until its plunger hits a mechanical stop and flow suddenly ceases. Gibson was unable to find a British manufacturer for his newly designed stopped-flow because the companies he approached were all of the opinion that the demand wouldn't be great enough [1]. The first commercial stopped-flow instrument, based on Gibson's design, was produced in 1965 by the US company Durrum Inc. (a predecessor of the present Dionex), based in Palo Alto, CA. Now, companies in the United Kingdom (TgK Scientific, Applied Photophysics), the United States (Update Instruments, KinTek Corporation, On-Line Instrument Systems), and France (Bio-Logic Science Instruments) are all producing stopped-flow instruments. Although they all have their differences, the principles of their flow cells are fundamentally based on Gibson's design.

7.2.2 Rapid Data Acquisition

Another crucial aspect for any stopped-flow instrument, and for any other rapid-reaction technique (e.g., temperature jump or voltage jump), is rapid data acquisition. It doesn't matter how short the mixing time of a stopped-flow instrument is if the

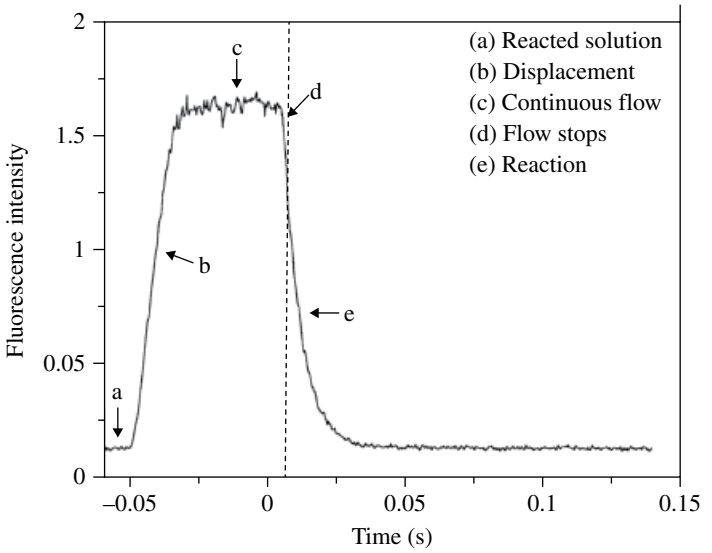

FIGURE 7.2 A typical stopped-flow fluorescence trace. Reproduced from Ref. 2 by permission of Oxford University Press.

data can't be captured at a sufficiently high rate to allow the calculation of rate constants for the system under study. In the absorbance- or fluorescence-based mode of detection, a photomultiplier is used to detect the change in light intensity caused by the reaction. The current output of the photomultiplier is then converted into a voltage by measuring the potential difference that the current produces across a known load resistor. Up until the 1980s, the transient changes in the voltage signal were commonly recorded by inputting the photomultiplier output into the voltage port of an analogue storage oscilloscope. Analysis of the data was then carried out by photographing the kinetic traces captured on the screen of the oscilloscope using a Polaroid camera, manually digitizing the data from the photographs and fitting to one or more exponential time functions to obtain an observed rate constant.

As with virtually any other instrument-based experimental technique, stopped flow has benefitted greatly by the revolution in electronic and computer technology. Storage oscilloscopes are now a thing of the past. In modern stopped-flow instruments, the analogue voltage signal coming from the photomultiplier is input directly into a computer, which converts it into a digital signal via an analogue-to-digital converter board. The experimental traces can then be directly averaged and fitted at the computer, reducing the time necessary for data analysis immensely.

An example of a typical stopped-flow fluorescence trace is shown in Figure 7.2. In this example, we have arbitrarily chosen a reaction that causes a decrease in fluorescence, but the principles are the same for a reaction causing an increase in fluorescence. In stopped-flow measurements, it is common practice to sometimes record the fluorescence level before the actual experiment starts. This is known as a "pre-trigger." In any stopped-flow experiment, it is necessary first to prime the flow circuit by flushing

through with small volumes of the two reactants so that any solutions remaining from an old experiment or from cleaning are removed. Therefore, an experiment starts with reacted solution already in the observation chamber. This corresponds to a low level of fluorescence in the experiment shown in Figure 7.2. Once the actual experiment starts and the drive syringes are pushing reactant solution into the observation chamber, the reacted solution is displaced and the fluorescence rises due to the high fluorescence of the unreacted solution in the example. After a short time, the rate at which fresh high fluorescent reactant enters the observation chamber via the drive syringes equals the rate at which the fluorescence decreases by the two solutions reacting with one another, and the fluorescence level reaches a plateau region. This is the continuous-flow region of the measurement. Once the stopping syringe is completely filled and the flow suddenly stops, no more fresh solution enters the observation chamber, and the fluorescence decays due to the reaction. This is the stopped-flow region, which is used for data fitting to obtain the observed rate constant of the reaction.

7.2.3 Dead Time

One of the most important considerations for any stopped-flow instrument is what's known as the "dead time." In Figure 7.1b, it can be seen that the point of observation is not at the point where the two solutions first meet. To obtain reliable measurements of rate constants, it's important that the two reactants be completely mixed before observing the reaction. Therefore, the observation chamber of the flow circuit must be positioned a certain distance further along the flow system. This means that solution reaching the observation chamber has already been reacting for a short period of time before observation commences. The term dead time is used because no data is captured during this initial part of the reaction. Of course, the magnitude of the dead time depends on the flow velocity, but typically for research-grade commercial stopped-flow instruments, the value is around 1–2 ms.

For very fast reactions, it could be the case that the entire reaction is over during the dead time. In this case, stopped-flow measurements would just yield a horizontal flat line, that is, a constant fluorescence level, and to obtain kinetic information on the system, one would have to resort to a different experimental technique with a higher time resolution, for example, a chemical relaxation method such as temperature jump. However, fast reactions that involve a very large change in fluorescence can still be measured using stopped flow if a sufficiently large change in fluorescence can still be observed following the dead time. This is demonstrated in Figure 7.3. As reactions become faster, more and more of the total amplitude of the fluorescence change is lost during the dead time. However, for any exponential decay or rise of the fluorescence due to a first order or pseudo first-order reaction, the same time constant or observed rate constant characterizes the entire curve. Therefore, even if only the tail of the reaction can be captured, this could be enough to analyze the data and determine the rate constant.

From the discussion just presented, it would seem that the shorter the dead-time, the better. However, if the dead time is too short, then it could be the case that the solutions are still incompletely mixed when they enter the observation cell. Then,

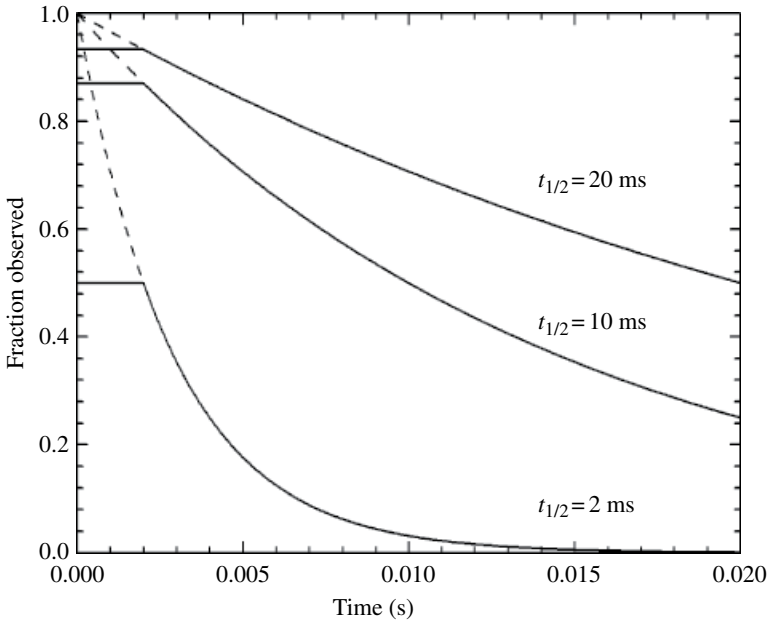

FIGURE 7.3 Stopped-flow fluorescence traces showing the increasing proportion of the total amplitude lost during the dead time as the speed of the reaction increases. The dead time was assumed to be 2 ms. For first-order reactions with half-lives of 20 ms, 10 ms, and 2 ms, the percentages of the reaction amplitude lost in the dead time are 6.7%, 13%, and 50%, respectively. Used with permission from TgK Scientific Ltd., Bradford on Avon, United Kingdom.

as described previously, the rate constant values obtained from the data analysis would actually be underestimates of the true values. Therefore, the value of the dead time of an instrument is a compromise. It must be sufficiently long to ensure complete mixing before observation starts but sufficiently short that not too much of the reaction is lost.

Experimental systems for measuring the dead time of a stopped-flow instrument in the fluorescence and absorbance modes and for testing the efficiency of mixing are described by Eccleston et al. [2].

7.3 COVALENT VERSUS NONCOVALENT FLUORESCENCE LABELING

As described previously, rapid-reaction flow methods in the solution phase were first developed to study the interaction of globin proteins such as hemoglobin and myoglobin with small ligands. These proteins contain intrinsic chromophores, porphyrins, whose UV/visible absorbance spectra are sensitive to ligand binding. Therefore, it is obvious that the first stopped-flow and continuous-flow measurements were carried out using the absorbance mode of detection. However, absorbance is a relative

measurement. It requires the determination of both the intensity of the incident and the transmitted light. In contrast, fluorescence is an absolute measurement. It just requires the measurement of the total light intensity emitted by a sample on its irradiation with exciting light. Therefore, fluorescence is inherently a much more sensitive mode of detection than absorbance. Just as stopped-flow arose out of continuous-flow driven by a desire to limit the expenditure of precious protein material, fluorescence-based stopped-flow is a logical choice over absorbance-based stopped-flow if one wishes to reduce protein consumption even further, which most researchers do.

7.3.1 Intrinsic Fluorescence

Most proteins contain one or more fluorescent tryptophan residues. Therefore, there is no need to label the protein if a reaction which one is interested in involves a sufficiently large change in tryptophan fluorescence. This is the most desirable situation because any extrinsic probe has the potential to influence protein activity in some way. By observing tryptophan fluorescence, one can study the protein in its native state. Fluorescence-based stopped-flow measurements are, therefore, most commonly performed using excitation in the ultraviolet region, because tryptophan can be selectively excited (over phenylalanine and tyrosine) in the wavelength range 295–305 nm [14]. The wavelength maximum of the fluorescence emission spectrum of tryptophan is sensitive to the polarity and the hydrogen-bonding capacity of its surrounding solvent. Therefore, protein conformational changes that alter the exposure of tryptophan to aqueous surroundings can be expected to show significant changes in tryptophan fluorescence intensity. For this reason, stopped-flow studies based on tryptophan fluorescence are extensively used in protein folding investigations, a topic of great current interest.

However, not all proteins containing tryptophan exhibit large changes in their fluorescence when they undergo conformational changes. It could be the case that some tryptophan residues are so deeply buried within the protein matrix that a conformational change doesn't significantly alter the exposure of the residue to water or the polarity of its surroundings. An example of the use of tryptophan fluorescence to probe the mechanism of an ion pump is the study of Karlish and Yates [15] who used stopped-flow to investigate the kinetics of conversion of membrane-bound Na^+,K^+-ATPase between its Na^+-selective E1 and K^+-selective E2 conformational states. However, the amplitudes of the fluorescence changes they observed were only around 2% at most, which made analysis difficult, particularly at high ATP concentrations when the reaction becomes faster. Therefore, in many cases, it is necessary to use extrinsic fluorescent probes to improve the signal-to-noise ratio or to obtain additional information not obtainable using tryptophan fluorescence.

Apart from the amplitude of the signal, other important considerations when choosing whether to use intrinsic protein fluorescence or an extrinsic probe are the wavelengths of excitation and emission. If one uses UV irradiation, the potential problem of components in the reaction mixture other than the target fluorophore absorbing either the exciting light or the emitted fluorescence, and thereby quenching the fluorescence signal, is greater than if one uses longer wavelength visible

light. This problem was a limiting factor in the study of Karlish and Yates [15] in which the overlap between the absorbance spectrum of ATP and the emission spectrum of tryptophan prevented measurements being carried out up to a saturating ATP concentration.

7.3.2 Covalently Bound Extrinsic Fluorescent Probes

There are a large number of fluorescent molecules that can be used to covalently label proteins and probe protein activity. For a more comprehensive coverage of the range of probes available, we suggest the reader consult other sources [14, 16]. Here, we concentrate on some basic principles and selected examples to illustrate the potential advantages and disadvantages of different probes.

One of the most commonly used fluorescent labels is fluorescein. A linking agent is needed in order to attach this probe to an amino acid residue of a protein. Three of the most widely used linkers are iodoacetamide, maleimide, and isothiocyanates. Iodoacetamides and maleimides attack sulfhydryl groups and hence target cysteine residues of the protein. Isothiocyanates attack amines and hence target basic amino acid residues such as lysine and arginine. Because of the problem of small tryptophan fluorescence amplitudes and quenching via ATP described previously, Karlish [17] turned to labeling of the Na^+,K^+-ATPase by fluorescein-5′-isothiocyanate (FITC), which labels lysine-501 of the protein [18]. Faller et al. [19] also used FITC to label the H^+,K^+-ATPase, a P-type ATPase related to the Na^+,K^+-ATPase, which is responsible for the acidification of the stomach. Both Karlish [17] and Faller et al. [19] were able to use the FITC-labeled enzymes to follow via stopped-flow the kinetics of conformational changes of these ion pumps when they underwent conformational changes. However, a major disadvantage of FITC labeling for kinetic studies is that the probe blocks the ATP binding [20]. Therefore, no kinetic studies of reactions activated by ATP can be carried out.

Labeling of the Na^+,K^+-ATPase by fluorescein, but using the iodoacetamide linker rather than isothiocyanate, was first introduced by Kapakos and Steinberg [21]. They found that the fluorescein label (5-IAF) showed significant changes in its fluorescence when the protein interacted with the ligands Na^+, K^+, and ATP, but, in contrast to FITC, the ATPase activity was unaffected. Subsequently, Steinberg and Karlish [22] were able to use 5-IAF-labeled enzyme to study the kinetics of the $E2 \rightarrow E1$ conformational transition via stopped-flow and show that the rate of the transition reached a saturating level at high ATP concentrations. This study would have been impossible using FITC because of blockage of the ATP site by the label. These studies demonstrate the importance of the site of labeling for any kinetic investigations of membrane protein mechanisms employing covalent fluorescent labeling.

Other covalent fluorescent labels that have been used to study reactions of the Na^+,K^+-ATPase include *N*-[*p*-(2-benzimidazoyl)phenyl]maleimide (BIPM) [23] and tetramethylrhodamine-6-maleimide (TMRM) [24]. Similar to 5-IAF, BIPM was found to have no effect on either Na^+,K^+-ATPase or H^+,K^+-ATPase activity and has been used in stopped-flow studies of both of these enzymes [23, 25]. The TMRM labeling [24] was carried out using mutations to the native Na^+,K^+-ATPase so that

only a single cysteine residue was available for reaction with TMRM. This form of site-directed fluorescent labeling was pioneered in the laboratories of Isacoff [26] and Bezanilla [27] for use on the Shaker K^+ channel. Although this is a powerful method for localizing amino acid residues involved in conformational changes of membrane proteins, a disadvantage is that both the mutations to the proteins and the TMRM labeling can modify the rate constants relative to the native enzyme [24]. Site-directed TMRM labeling and its use in combination with the voltage clamp technique is the subject of Chapter 4.

7.3.3 Noncovalently Bound Extrinsic Fluorescent Probes

Noncovalently bound extrinsic fluorescent probes have the advantage that they don't chemically modify the protein under investigation. However, they can still modify protein function. Therefore, one needs to test carefully if this is the case and possibly take any inhibition into account in data analysis or experimental design.

A noncovalent probe that has been extensively used together with stopped-flow to study reactions of the Na^+,K^+-ATPase is eosin Y (often simply referred to as eosin). Eosin was first used in this application by Skou and Esmann, who initially attempted to covalently attach it to the enzyme via a maleimide linkage [28]. However, in the course of their studies, they found that even noncovalently bound eosin responded to the addition of Na^+ or K^+ to the enzyme with a fluorescence change [29]. By comparing the interaction of the enzyme with ATP in the presence of eosin, they were able to conclude that eosin binds directly in the enzyme's ATP binding site. Unfortunately, the blockage of the ATP binding site by eosin precludes its use in any kinetic studies of partial reactions of the enzyme involving ATP hydrolysis. However, Skou and Esmann [30] used it via stopped-flow to study the rates of transition between the E1 and E2 conformational states of the enzyme, which is associated with a change in affinity of the enzyme for eosin, just as there is for ATP. By stopped-flow measurements, in which he mixed an enzyme–eosin complex with ADP or ATP, Esmann [31] was also able to determine rate constants for nucleotide displacement from the enzyme.

Perhaps the most useful noncovalently bound fluorescent probes for studying the kinetics of ion pumps are voltage-sensitive probes. All electrogenic ion pumps, such as the Na^+,K^+-ATPase, transport net charge across the membrane. The current they generate thus builds up a transmembrane electrical potential difference across the membrane, which can be sensed by some voltage-sensitive dyes. The conformational changes they undergo during ion transport can also cause local changes in electric field strength within the membrane. These can be sensed by other voltage-sensitive dyes. Because all voltage-sensitive dyes respond to electrical field strength changes within or across membranes, they can indirectly react to the activity of pumps, channels, or transporters. There is no need for them to interact directly with any membrane protein to be able to kinetically follow its activity. However, this doesn't exclude the possibility that some voltage-sensitive dyes may also interact directly with membrane proteins as well as with the surrounding lipid phase. The remaining sections of this chapter are devoted to voltage-sensitive dyes exclusively.

7.4 CLASSES OF VOLTAGE-SENSITIVE DYES

Voltage-sensitive dyes are commonly divided into two classes based on their response times to rapid changes in the transmembrane voltage: fast dyes and slow dyes. Fast dyes have response times of less than milliseconds. Dyes belonging to this class include styrylpyridinium and annellated hemicyanine dyes, merocyanine dyes, and 3-hydroxychromone dyes. Slow dyes have response times greater than milliseconds. Dyes belonging to this class include cationic carbocyanine and rhodamine dyes and anionic oxonol dyes. The response times of the dyes determine the type of information that can be gained on the mechanisms of pumps, channels, or transporters.

Readers wishing to gain more detailed information on the range of dyes available, their response mechanisms, and their photostability and phototoxicity are referred to a recent review [32]. Here, we concentrate predominantly on aspects related to their application in determining the mechanism of ion pumps.

7.4.1 Slow Dyes

Slow dyes (see Fig. 7.4) respond via a movement of dye across the entire membrane. When the transmembrane potential difference changes, slow dyes redistribute themselves across the membrane. For example, if the cytoplasm becomes more positive relative to the extracellular fluid, anionic dyes accumulate in the cytoplasm. Similarly, if the cytoplasm becomes more negative relative to the extracellular fluid, cationic dyes accumulate in the cytoplasm. Any accumulation of dye in the cytoplasm also perturbs the partitioning of dye between the aqueous and membrane phases. Under most circumstances, the total intracellular volume of a suspension of cells is much smaller than the total volume of the extracellular fluid. Therefore, an accumulation of dye in the cytoplasm is also expected to result in an increase in dye bound to the membrane. If the fluorescence of dye bound to the membrane and in an aqueous environment is different, the dye responds to the change in transmembrane potential with a fluorescence change.

Because slow dyes respond to the transmembrane potential difference, they can only be used to study pumps, channels, or transporters in intact cells, intact organelles, or reconstituted into synthetic lipid vesicles. The greater than millisecond response time of slow dyes is due to the requirement of their mechanism that they move across the entire membrane. Many individual partial reactions in the mechanisms of pumps, channels, or transporters are much faster than this. Therefore, although slow dyes are useful in detecting changes in transmembrane potential due to changes in the activity of ion-transporting membrane proteins, their application in kinetic studies of such proteins is limited.

It is, however, possible to use slow dyes to follow the steady-state activity of electrogenic ion pumps. When reconstituted into lipid vesicles, the Na^+,K^+-ATPase causes a change in transmembrane potential because it pumps 3 Na^+ ions in one direction and 2 K^+ ions in the other direction across the membrane, that is, there is a net transport of one positive charge per enzyme turnover per ATP molecule hydrolyzed. Because the mechanisms of ion pumps must involve substantial

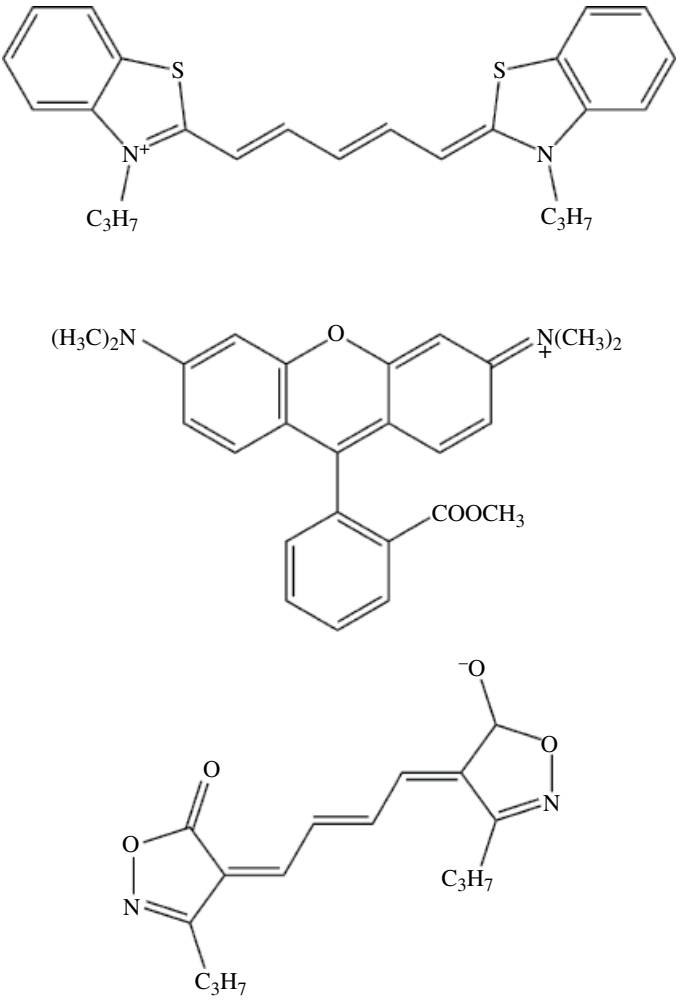

FIGURE 7.4 Classes of slow voltage-sensitive fluorescent dyes. From above, examples of voltage-sensitive carbocyanine, rhodamine and oxonol dyes are shown, respectively. Reprinted from Ref. 32 with kind permission from Springer Science and Business Media.

conformational changes in order to transport ions against a concentration gradient, the ion fluxes they produce across membranes are generally much lower than those of ion channels. Maximum turnovers in the range 10–100 s^{-1} at normal physiological temperatures are typical. A stopped-flow investigation [33] of the interaction of the slow dye oxonol VI with lipid vesicles showed that it responds to changes in transmembrane potential in less than a second. This is sufficiently fast that it could be used in a fluorimeter to kinetically follow the build-up of the transmembrane potential by the Na^+,K^+-ATPase in lipid vesicles [34, 35]. Much more detailed kinetic information can, however, be obtained using fast dyes. We therefore now turn our attention to them.

7.4.2 Fast Dyes

Fast dyes (see Fig. 7.5) respond to changes in intramembrane electric field strength via a reorientation of the dye molecule within the membrane and/or a redistribution of the dye's electrons within its chromophore. The amplitudes of their fluorescence responses are in general significantly smaller than those of slow dyes, but for kinetic studies, this disadvantage is far outweighed by the advantage of their much faster response time.

For research on the kinetics of ion pumps, by far, the most widely used fast dyes are those based on the aminostyrylpyridinium chromophore, in particular the dye RH421 (see Fig. 7.5). The development of this chemical class of dyes arose out of quantum mechanical calculations by Loew et al. [36], which led to the prediction that the aminostyrylpyridinium chromophore should be ideally suited to respond to changes in membrane potential via an electrochromic mechanism. Electrochromism can be defined as an electric-field-induced wavelength shift in the UV/visible absorbance spectrum or fluorescence excitation spectrum of a molecule. For such a wavelength shift to occur, the molecule must undergo a large charge shift on excitation, that is, the electron distribution must be very different in the excited state compared to the ground state. This is indeed the case for the aminostyrylpyridinium chromophore (see Fig. 7.6). The reason for the electrochromic wavelength shift is that any local electric field within the membrane causes different degrees of stabilization or destabilization of the ground and excited states of membrane-bound dye. Thus, the energy gap between the ground and excited states and the wavelength of maximum absorbance, λ_{max}, are electric-field-dependent. Because this mechanism only involves a redistribution of the probe's electrons, the response is expected to be very fast, that is, on the femtosecond timescale. If one uses fixed wavelengths of excitation and emission, the electrochromic shift in the excitation spectrum would cause either an increase or decrease in the observed fluorescence intensity depending on whether the excitation wavelength used is on the low- or high-wavelength side of λ_{max}.

After theoretically predicting the potential of the aminostyrylpyridinium chromophore, Loew and collaborators went ahead and synthesized a range of dyes based on the chromophore and successfully demonstrated their electrical responses in both model membrane systems and intact cells [37–41]. Probably, the most widely used and most well-known dye originating in Loew's laboratory at the State University of New York at Binghamton is now di-8-ANEPPS, which contains a naphthyl derivative of the aminostyrylpyridinium chromophore. The name is an acronym of its chemical name.

Another important laboratory that contributed to the development of fast response dyes is that of Grinvald [42, 43] at the Weizmann Institute in Rehovot, Israel. The dyes developed there are known as RH dyes, but they are also based on the same aminostyrylpyridinium chromophore used in Loew's laboratory. The RH designation derives from the name of Grinvald's collaborator Rina Hildesheim, who actually synthesized the dyes. The most well-known dye originating in their laboratory is RH421 (see Fig. 7.5).

Although the ANEPPS and RH dyes were synthesized with an electrochromic mechanism in mind, testing on both model membrane systems and cell preparations

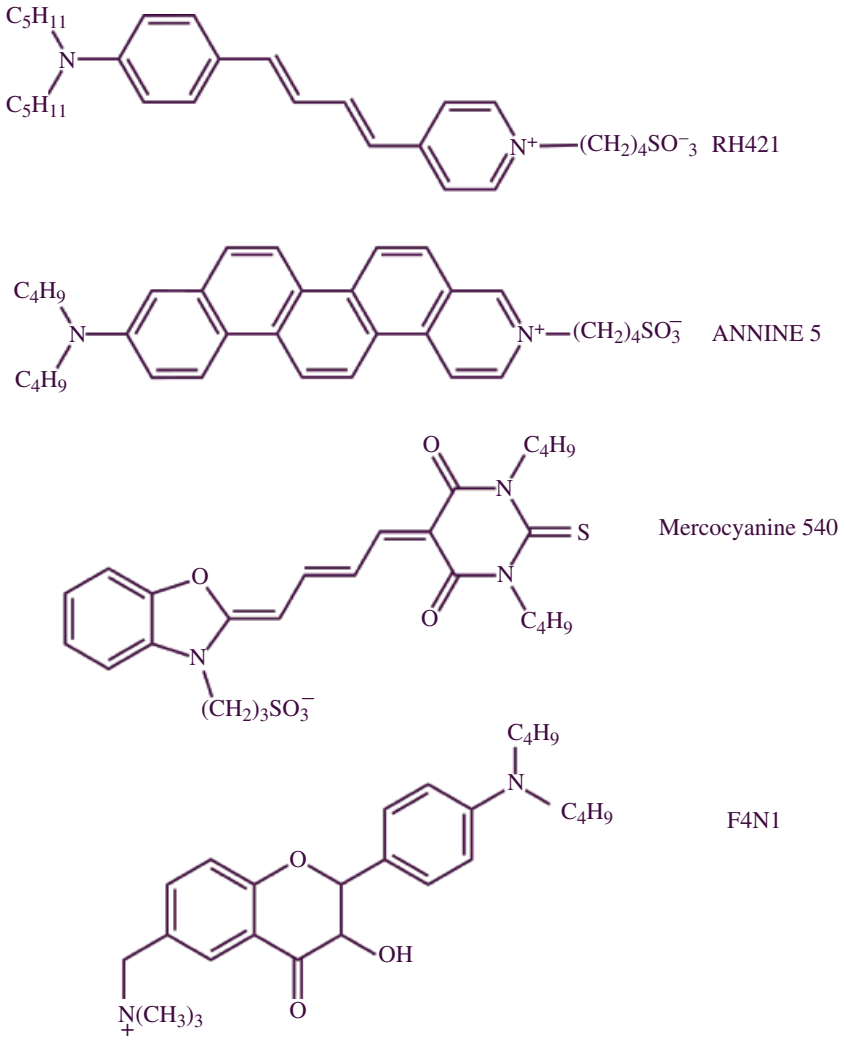

FIGURE 7.5 Classes of fast voltage-sensitive fluorescent dyes. From above, examples of voltage-sensitive styrylpyridinium, annellated hemicyanine, merocyanine and 3-hydroxychromone dyes are shown, respectively. Reprinted from Ref. 32 with kind permission from Springer Science and Business Media.

suggests [39, 40, 44, 45] that for many of them, their electrical responses are not purely electrochromic. For example, Fluhler et al. [40] stated that "The impressive sensitivity of RH421 in neuroblastoma cells is clearly too large to be attributable to electrochromism." The same conclusion was reached from studies on model membrane systems and Na^+,K^+-ATPase-containing membrane fragments [44, 45]. In these systems, evidence for a reorientation/solvatochromic contribution to the overall response of RH421 was found. From measurements in chloroform, RH421 was

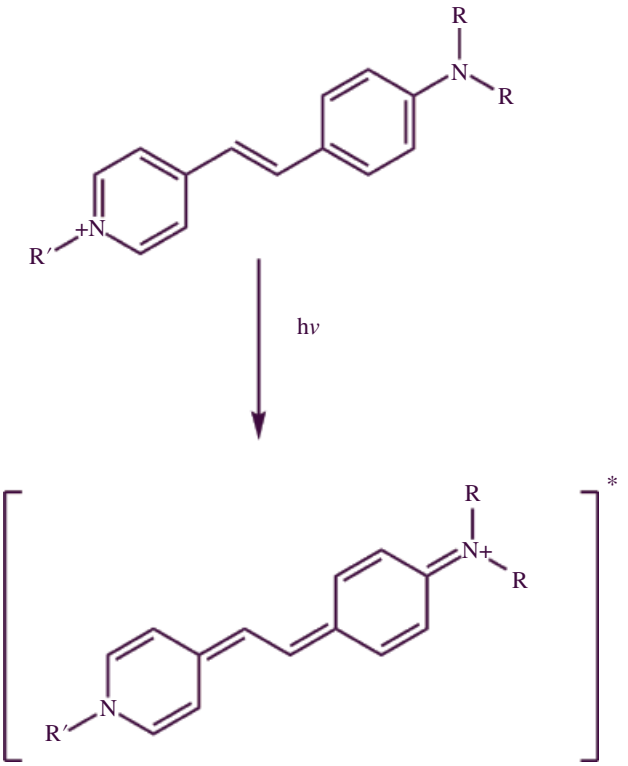

FIGURE 7.6 Electron redistribution occurring during excitation of the aminostyrylpyridinium chromophore. Reprinted from Ref. 32 with kind permission from Springer Science and Business Media.

found [44] to have a dipole moment of 12 (±2) Debye. Any change in the transmembrane electrical potential would cause a change in electric field strength within the membrane, which would result in the reorientation of the RH421 dipole within the membrane. Lipid membranes are known to possess a sharp polarity gradient on going from the lipid headgroup region into the hydrocarbon interior of the membrane. Because of their amphiphilic structure, the dyes are thought to reside precisely in this region of sharply changing polarity. Therefore, any small reorientation of the dye population in the membrane brought about by a change in the transmembrane electric field could significantly change the average local polarity the dye molecules experience. This would cause a solvatochromic shift in their absorbance and fluorescence excitation spectra, because a change in polarity would differentially stabilize or destabilize the ground and excited states of the dye, just as a transmembrane electric field does. Furthermore, if the dye reorients so that the long axis of its chromophore becomes more perpendicular to the membrane surface, this would strengthen its interaction with the transmembrane field and yield a larger electrochromic shift. Thus, electrochromism and solvatochromism could work together to increase the overall response of a dye to the transmembrane electrical potential.

Apart from the chromophore, other important structural aspects of the RH and ANEPPS dyes are that they each possess a negatively charged sulfonate group attached via an alkyl chain linker to the pyridinium ring and two hydrocarbon chains attached to the anilino nitrogen (see, e.g., RH421 in Fig. 7.5). These groups have two important purposes. Firstly, the hydrophilic sulfonate group acts as an anchor fixing that end of the molecule at the interface of the membrane with the adjacent aqueous medium. The hydrophobic hydrocarbon chains, on the other hand, insert deeply into the hydrophobic interior of the membrane. Thus, together, the sulfonate group and the hydrophobic chains tend to orient the dye molecules so that the major component of their chromophore is aligned perpendicular to the membrane surface, which strengthens the interaction with the transmembrane electric field.

The other purpose of the sulfonate group is to prevent the dye from diffusing across the membrane [38, 40]. Measurements on synthetic bilayers have shown [38] that the direction of the fluorescence change on application of a transmembrane electric field reverses if dye is added to the inside of the bilayer rather than the outside. This is because the polarity of the field is opposite for dye in the internal and external leaflets of the bilayer. Thus, an inside positive electrical potential destabilizes the positive charge on the pyridinium nitrogen of dye in the inner leaflet, whereas, if dye molecules are in the outer leaflet, positive charges on their pyridinium nitrogens are stabilized. If dye were present in equal concentrations in both leaflets, their electrochromic responses would, therefore, exactly cancel. The same applies to a reorientation/solvatochromic response mechanism. When using the dyes to detect changes in transmembrane electrical potential in cells, cell organelles, or closed vesicles, it is important, therefore, to just add the dyes to one side of the membrane. The dyes may not be completely impenetrable to the membrane, but the sulfonate group ensures that any flip-flop that does occur is very slow.

7.5 MEASUREMENT OF THE KINETICS OF THE Na^+,K^+-ATPase

Voltage-sensitive fast dyes have been most widely used to image the transmembrane potential and electrical activity of excitable cells, in particular neurons [46–48]. Here, we consider a more specific application, that is, fundamental research on the mechanisms of individual ion-transporting membrane proteins. RH and ANEPPS dyes have been applied in investigations of the mechanisms of a number of ion pumps, for example, the Na^+,K^+-ATPase [49–51], the fungal plasma membrane H^+-ATPase [52–54], bacteriorhodopsin [55], the gastric H^+,K^+-ATPase [51, 56], and the sarcoplasmic reticulum Ca^{2+}-ATPase [57].

The first work on the mechanism of an ion pump using fast styrylpyridinium dyes (RH160 and RH421) was that of Klodos and Forbush [49, 50] on the Na^+,K^+-ATPase. They used open membrane fragments from dog kidney containing a high surface density of Na^+,K^+-ATPase molecules for their measurements. Because the fragments were open on all sides to the surrounding electrolyte solution, there could be no transmembrane potential initially present, and after activation of the Na^+,K^+-ATPase by the addition of ATP, the pumping of Na^+ and K^+ ions across the membrane by the protein

couldn't produce any transmembrane potential. Klodos and Forbush [49, 50], therefore, concluded that the fluorescence changes they observed were due to local electric field effects within the membrane or a direct interaction with the Na^+,K^+-ATPase.

Nagel et al. [53, 54] also used an open membrane fragment system for their studies on the fungal H^+-ATPase with the dye RH160. They observed that the fluorescence responses that occurred on addition of ATP or the inhibitor vanadate were inconsistent with a purely electrochromic mechanism of the dye, that is, in accord with other studies on the mechanisms of styrylpyridinium dyes described in Section 7.4.2.

In the following sections, we will limit ourselves to one particular case study: the use of the dye RH421 in stopped-flow investigations of the mechanism of the Na^+,K^+-ATPase. In the context of this case study, we explain all the important practical considerations relevant to the use of any fast voltage-sensitive dye in kinetic studies of ion pumps. We have chosen this example because up to now, it is the most extensively studied system and the experimental approaches required to obtain the best possible data are already well established.

7.5.1 Dye Concentration

One of the simplest but most important considerations in any kinetic study using fast dyes is what dye concentration to use. Frank et al. [58] found that micromolar concentrations of RH421 inhibit the steady-state activity of the Na^+,K^+-ATPase. Subsequently, using several different time-resolved experimental techniques, Kane et al. [59] were able to localize the reaction step of the enzyme inhibited by the dye. They found that there was no effect of micromolar concentrations of RH421 on the kinetics of phosphorylation of the Na^+,K^+-ATPase and concluded that RH421 inhibits a reaction downstream from phosphorylation, possibly the E1P to E2P conformational transition of the enzyme, which is involved in deocclusion of Na^+ and its release to the extracellular medium. For future kinetic studies, the important practical point is that the RH421 concentration should be kept in the submicromolar range to avoid any dye-induced inhibition and obtain true values of any rate constants.

There are two possible origins for RH421-induced inhibition of the Na^+, K^+-ATPase. One is a direct binding of RH421 to the protein; another is indirect through dye binding to the lipid phase of the membrane. First, we will consider direct protein binding. It is known that RH421 can bind to globular proteins, for example, bovine serum albumin [60] and ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) [58], and to polyamino acids, for example, poly(L-lysine), poly(L-arginine), and poly(L-tyrosine) [58]. On the other hand, no spectral changes have been detected when poly(L-glutamic acid), poly(L-aspartic acid), or poly(L-serine) is added to the dye [58]. The observed interactions with poly(L-lysine) and poly(L-arginine) suggest that one mode of dye binding to proteins is via interaction between the negatively charged sulfonate group of the dye and the positively charged side chains of the basic amino acid residues lysine and arginine. Schwappach et al. [60] concluded that RH421 interacts with the protein as well as with the lipid bilayer. This conclusion was based on two findings: (i) resonance energy transfer between the dye and fluorescence probes on the enzyme and (ii) the observation of biexponential fluorescence

lifetime decays of RH421 bound to Na^+,K^+-ATPase-containing membrane fragments. However, more extensive fluorescence studies of the dye have shown that its photochemistry is complex, and multiexponential fluorescence decays are observed even for dye in homogeneous solvents [45, 61–63]. Therefore, the biexponential nature of the fluorescence lifetime decays reported by Schwappach et al. [60] cannot be used as evidence for direct binding to the Na^+,K^+-ATPase. Recently, using a cytoplasmic fragment of the Na^+,K^+-ATPase, the group of Kubala (unpublished results) found evidence that at micromolar concentrations, RH421 could interact directly with the ATP binding site, that is, similar to eosin. However, based on the kinetic measurements of Kane et al. [59], an inhibition of the Na^+,K^+-ATPase in membrane fragments due to a direct competition between ATP and RH421 for binding to the same site seems unlikely because this would significantly slow the kinetics of ATP phosphorylation, in contradiction to experimental observations. It's possible that in the presence of both protein and lipid membrane, the dye may bind preferentially to the lipid membrane rather than to the ATP binding site, in part perhaps because much more lipid is available for binding than ATP sites. Nevertheless, this doesn't exclude the possibility that RH421 may bind elsewhere on the Na^+,K^+-ATPase and lead to inhibition of pumping activity of the protein in membrane fragments.

The second possible cause of dye-induced Na^+,K^+-ATPase inhibition is via an effect from dye bound to the lipid phase of the membrane. For any dye to be able to detect changes in intramembrane electric field strength, it must have either a net charge or be zwitterionic, as is the case with RH421. Therefore, charges on the dyes could potentially influence the kinetics of charge movements associated with protein activity. Via electrical measurements on planar lipid bilayers, Malkov and Sokolov [64] showed that the dyes RH421, RH237, and RH160 all increase the electric field strength in the boundary layer of the membrane because of the positive charge on their pyridinium rings. The negatively charged sulfonate group presumably has no effect because it would be localized in the high dielectric constant medium of the adjacent aqueous phase. It has also been found [65] that the electric field produced by bound dye molecules in the membrane affects neighboring dye molecules and shifts their fluorescence excitation spectrum. If the intramembrane electric field strength produced by dye binding to the membrane becomes too high, it's possible that this affects the kinetics of the Na^+,K^+-ATPase. According to calculations of Malkov and Sokolov [64], the fields produced by the dyes are likely to have only a minor effect on ion conduction through the center of the protein because of screening from the intervening protein mass. There could, however, be other reactions of the Na^+,K^+-ATPase where there is a stronger effect.

Another important aspect to the question of the dye concentration to use is the effect that the dye has on its own response. Even at concentrations below 1 μM, where one would expect negligible inhibition of the Na^+,K^+-ATPase, Frank et al. [58] have found that the fluorescence response of RH421 to the addition of ATP to the enzyme decreases rapidly with increasing dye concentration (see Fig. 7.7). Of course in all experiments, the dye concentration needs to be high enough that one can detect its fluorescence, but Figure 7.7 indicates that the dye concentration should be as low as possible to achieve the maximum possible relative fluorescence change. In their

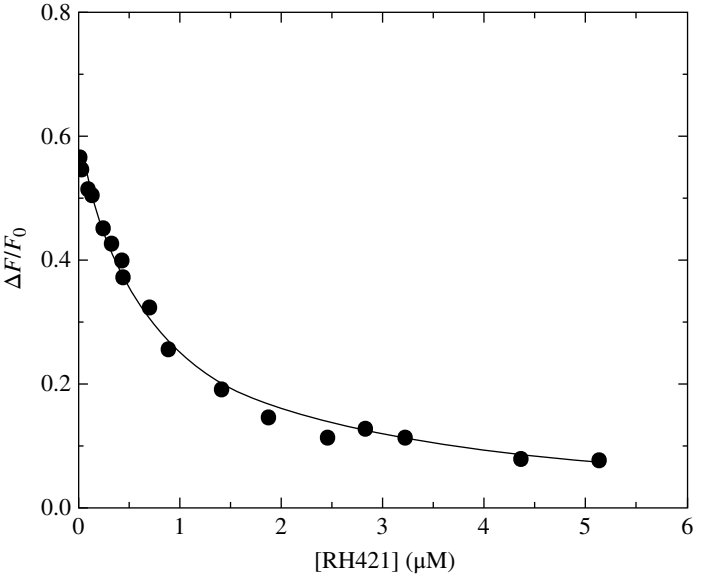

FIGURE 7.7 Decrease in the relative fluorescence change, $\Delta F/F_0$, of Na^+,K^+-ATPase-containing membrane fragments noncovalently labeled with RH421 as the concentration of the probe increases. The solid line represents a fit of the equation $\Delta F/F_0 = (\Delta F/F_0)_{max} \cdot (K/K + c)$ to the experimental data, where $(\Delta F/F_0)_{max}$ is the theoretical maximum relative fluorescence at infinite dilution of the dye, c is the total dye concentration, and K is the dye concentration at which the relative fluorescence change has dropped to half of its infinite dilution value. The values obtained from fitting were $(\Delta F/F_0)_{max} = 0.593$ (±0.009) and $K = 0.73$ (±0.04) μM. Reproduced from Ref. 58 with permission from Elsevier.

stopped-flow experiments in which they mixed the Na^+,K^+-ATPase-containing membrane fragments with ATP, Kane et al. [59] added 150 nM of RH421 to the protein before mixing. Because equal volumes from both drive syringes were mixed, the RH421 concentration in the stopped-flow observation cell was 75 nM after mixing. This is definitely low enough to avoid any inhibition of the protein's activity as well as to avoid any significant drop-off in the dye's response.

The reason for the drop in dye response with increasing dye concentrations isn't entirely clear at this stage. However, possible explanations have been proposed. It seems reasonable that if the dye is responding to changes in local electric field strength due to the activity of the Na^+,K^+-ATPase, then dye molecules close to a protein molecule should produce a larger response than those located further away. This explains the much lower response of RH421 when used on Na^+,K^+-ATPase reconstituted into lipid vesicles [66] in comparison to measurements on Na^+,K^+-ATPase-containing membrane fragments, which have a very high protein density of up to 10^5 pumps μm^{-2} [67]. Dye molecules located in the membrane but at a large distance from protein molecules would produce a constant background fluorescence and decrease the fluorescence response in membranes with a low protein density relative to those with a high protein density. This by itself doesn't explain the

concentration-dependent drop in dye response observed in membrane fragments. However, if the dye binds preferentially in the vicinity of Na^+,K^+-ATPase molecules, as efficient RH421 quenching of the tryptophan fluorescence of Na^+,K^+-ATPase via resonance energy transfer suggests [58], then, as the dye concentration increases, the amount of dye binding at distances far from a Na^+,K^+-ATPase would gradually increase. Thus, the constant background RH421 fluorescence would increase and the relative fluorescence response to Na^+,K^+-ATPase activity would decrease.

Another reason or contributing factor to the concentration-dependent drop in dye response could lie in the electric field effect of dye molecules on one another [58], described previously in the context of Na^+,K^+-ATPase inhibition. Dye molecules in the membrane surrounding any other particular dye molecule would create a constant electric field strength, which would oppose any changes in local electric field strength arising from Na^+,K^+-ATPase activity. In effect, the dye molecules would polarize each other's electron clouds and dipoles and make it harder for any dye molecule to respond via either an electrochromic or reorientational/solvatochromic mechanism. Therefore, the largest fluorescence response would be expected at low dye concentrations where each dye molecule is isolated in the membrane, unaffected by the fields of any other dyes molecules, so that it responds completely to fields originating from the Na^+,K^+-ATPase alone.

7.5.2 Excitation Wavelength and Light Source

RH421 responds to a change in its local electric field strength with a shift in its fluorescence excitation spectrum (see Fig. 7.8). Therefore, if the local electric field strength within a membrane changes due to Na^+,K^+-ATPase activity or any other cause, there will be a certain excitation wavelength where the excitation spectra before and after the change cross. If one carried out stopped-flow measurements using this particular excitation wavelength, no change in fluorescence would be detected at all. Therefore, one needs to be careful in selecting an appropriate excitation wavelength.

In any system where the fluorescence excitation spectrum shifts, the highest relative changes in fluorescence are observed on the flanks of the spectrum, that is, on the low-wavelength (blue) edge or the high-wavelength (red) edge of the spectrum. For a number of reasons, excitation on the red edge is preferable. Lower energy red-edge excitation is less likely to cause photochemical damage to the dye. It is also less likely to be absorbed by any other components in the reaction mixture, for example, buffers, and cause unwanted background fluorescence. Long wavelength excitation also decreases the amount of light scattering, which is an important consideration when using membrane systems.

The fluorescence intensity detected in any steady-state fluorometric technique such as stopped-flow fluorimetry depends on the steady-state proportion of dye molecules in the excited state relative to the ground state, which increases with the number of photons absorbed per unit time. Therefore, one needs a lamp with a high photon flux at the desired wavelength of excitation to detect a high fluorescence intensity. This is particularly important for stopped-flow measurements, where there

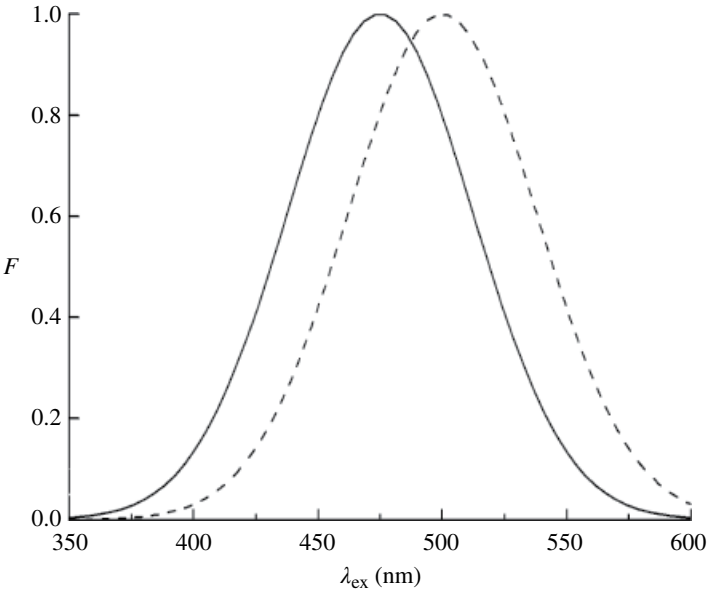

FIGURE 7.8 Shift in the normalized fluorescence excitation spectrum of RH421 expected on changing the intramembrane electric field strength. If the amount of positive charge within the membrane decreases, the fluorescence excitation spectrum of RH421 shifts to longer wavelengths, that is, from the solid curve to the dotted curve.

is a limited time over which the fluorescence is measured. In measurements of fluorescence spectra, many individual spectra can be recorded and averaged to increase the signal-to-noise ratio. However, in stopped-flow, an individual measurement is often over in less than a second. Therefore, one needs to be able to maximize the fluorescence intensity detected. This generally necessitates using an arc lamp, that is, xenon (Xe), mercury (Hg), or mercury–xenon (Hg–Xe), rather than a filament lamp such as a quartz–tungsten halogen (QTH) lamp. The output of QTH lamps is more stable than that of arc lamps, but their intensities are too low for fluorescence, except for measurements in the near infrared.

For the excitation of RH421 in stopped-flow measurements on the Na^+,K^+-ATPase, the authors have used Hg or Hg–Xe lamps. The advantage of these lamps is that they possess very intense lines corresponding to transitions in the electronic spectrum of Hg vapor. For RH421, there is a perfectly positioned line at 577 nm, which lies exactly on the red edge of the dye's excitation spectrum and is, thus, optimal for yielding large relative fluorescence changes.

7.5.3 Monochromators and Filters

The excitation wavelength can be selected by using an excitation grating monochromator. The transmission efficiency of a monochromator, however, varies with the wavelength of the transmitted light and depends on its construction. The wavelength

of maximum transmission is termed the "blaze wavelength" [14]. Because the most common application of stopped-flow fluorimetry involves the measurement of tryptophan fluorescence, commercial stopped-flow instruments are generally supplied with an excitation monochromator grating with a blaze wavelength in the ultraviolet region. However, for more efficient excitation of RH421 at 577 nm, it's desirable to use a grating with a blaze wavelength in the visible range. The authors use a grating with a blaze wavelength of 500 nm. This improves the signal-to-noise ratio of RH421 fluorescence transients, but it doesn't preclude measurements at lower excitation wavelengths for other applications.

To select the fluorescence emission wavelength range, in principle, it's possible to use an emission monochromator in front of the photomultiplier used for detection. However, fluorescence light is always lost on passage through a monochromator. Therefore, to increase the amount of fluorescence light detected, the authors prefer to use a colored glass cutoff filter, that is, a Schott RG665 filter, with 50% transmission at 665 nm. This allows all of the fluorescence above 665 nm to be collected. The large wavelength difference between excitation at 577 nm and emission at ≥665 nm ensures that very little scattered exciting light reaches the photomultiplier and the vast majority of the detected light is actual fluorescence.

If one wished, in principle, one could also use a combination of glass filters or an interference filter on the excitation side rather than a monochromator. This could further increase the intensity of the 577 nm light reaching the sample and increase the emitted fluorescence further. However, increasing the exciting light intensity is a two-edged sword, because it always carries with it the danger that one could at the same time increase the likelihood of photochemical reactions such as bleaching of the dye. Therefore, apart from increasing the efficiency of excitation, it is also worthwhile to consider the efficiency of light detection.

7.5.4 Photomultiplier and Voltage Supply

A voltage needs to be applied across the dynodes of the photomultiplier to amplify the signal for the photon flux of fluorescent light hitting the photocathode of a photomultiplier to produce a measurable output. The higher the applied voltage, the higher the signal (initially a photocurrent, but converted to a photovoltage via a resistor). However, a higher applied voltage also amplifies the noise. Therefore, to obtain the best signal-to-noise ratio, it is best to use the lowest possible applied voltage and use a photomultiplier with a particularly high sensitivity in the wavelength range of detection.

For the long wavelength detection of RH421 fluorescence at wavelengths ≥665 nm, it is desirable to use a photomultiplier tube with a high red sensitivity. A good choice is the Hamamatsu multialkali side-on R928 photomultiplier tube. With the high red sensitivity, the applied photomultiplier voltage can be reduced, reducing the signal noise, and the intensity of the exciting light can be reduced, reducing the danger of photochemical damage. The R928 tube still has excellent sensitivity down into the ultraviolet region, so that its use doesn't preclude other applications.

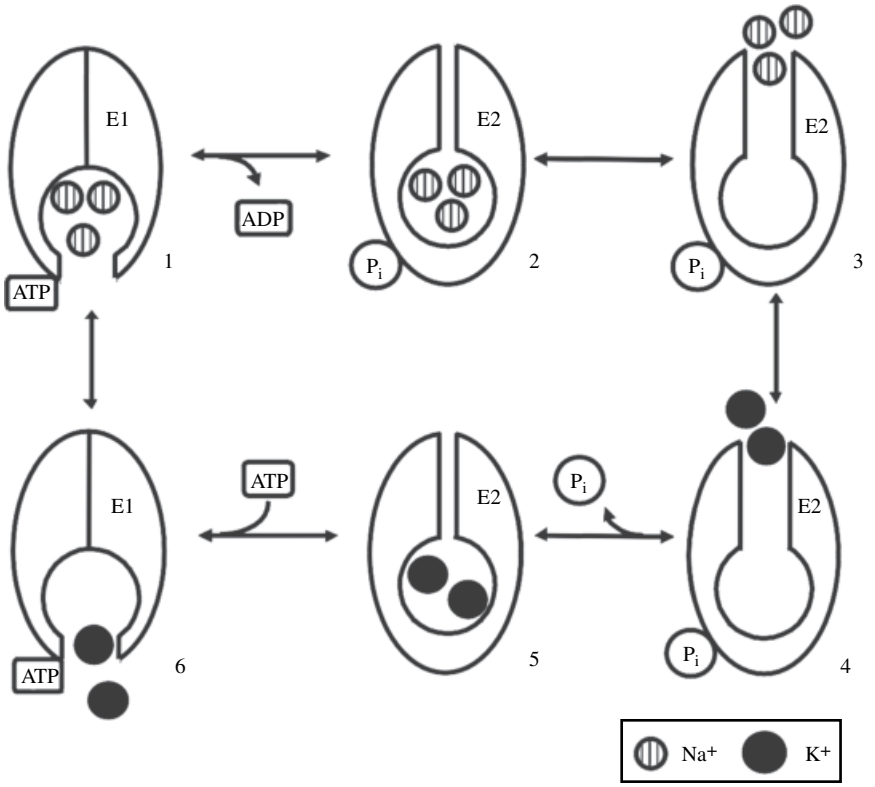

FIGURE 7.9 Albers–Post model of the Na^+,K^+-ATPase reaction cycle. The entire protein is embedded in the cell plasma membrane (not shown). The top of the protein interfaces with the extracellular medium, whereas the bottom of the protein interfaces with the cytoplasm of the cell. Thus, each cycle, in which one ATP molecule is hydrolyzed to inorganic phosphate P_i, involves the pumping of $3Na^+$ ions out of the cell and $2K^+$ ions in. Adapted from Ref. 68 with permission from Wiley.

7.5.5 Reactions Detected by RH421

The sequence of reaction steps that the Na^+,K^+-ATPase undergoes in pumping Na^+ and K^+ across the plasma membrane is described by the Albers–Post or E1–E2 cycle (see Fig. 7.9). Analogous reaction cycles but with different transported ions can be drawn for all other P-type ATPases. In principle, there are three ways in which stopped flow can be used together with RH421 to determine rate constants of reaction steps of this cycle:

a) Mixing Na^+,K^+-ATPase with ATP in the presence of Na^+ and Mg^{2+}, but in the absence of K^+. Reactions occurring: $E1(Na^+)_3 + ATP \rightarrow E1P(Na^+)_3 + ADP$ immediately followed by $E1P(Na^+)_3 \rightarrow E2P + 3Na^+$. Fluorescence of RH421 increases.

b) Mixing Na^+,K^+-ATPase with Na^+ with or without ATP, but in the absence of Mg^{2+} and K^+. Reactions occurring: $E2 \rightarrow E1(Na^+)_3$ or $E2ATP \rightarrow E1(Na^+)_3ATP$. Fluorescence of RH421 decreases.

c) Mixing Na^+,K^+-ATPase, pre-equilibrated with Na^+, Mg^{2+}, and ATP, with K^+. Reactions occurring: $E2P + 2K^+ \rightarrow E2P(K^+)_2$ followed immediately by $E2P(K^+)_2 \rightarrow E2(K^+)_2 + P_i$. Fluorescence of RH421 decreases.

In each of these different types of experiments, the Na^+,K^+-ATPase-containing membrane fragments must be premixed with RH421, added from an ethanolic stock solution, because incorporation of dye into the membrane fragments is a relatively slow process occurring over tens of seconds [69]. As soon as the dye is added to aqueous solution, it forms large aggregates, which need to disaggregate so that the dye can insert in monomeric form into the membrane. The Na^+,K^+-ATPase-containing membrane fragments noncovalently labeled with RH421 are added to one drive syringe and the substrates with which the enzyme is to be mixed are added to the second drive syringe. RH421 could, in principle, also be added to the second drive syringe to avoid dissociation of dye from the membrane fragments. However, because the dye binds strongly to the membrane, the amount of dissociation is likely to be very small, and the fluorescence of membrane-bound dye is far higher than that of dye in aqueous solution. Therefore, in practice, it seems unnecessary to include dye in the second syringe.

Examples of fluorescence transients obtained using RH421 in the first type of experiment are shown in Figure 7.10 [70]. In this study, Na^+,K^+-ATPase-containing membrane fragments noncovalently labeled with RH421 were equilibrated in a buffer containing Na^+ and varying concentrations of Mg^{2+} and then rapidly mixed with ATP. K^+ was completely excluded from the solutions in both drive syringes. The exclusion of K^+ inhibits dephosphorylation of the enzyme and after mixing with ATP causes it to accumulate in the E2P state, which is associated with a high fluorescence of RH421. The transients shown here demonstrate the large amplitudes of the fluorescence changes that can be obtained, that is, relative fluorescence changes of up to 400%. The measurements were made using Na^+,K^+-ATPase-containing membrane fragments purified from shark rectal glands. In this case, the protein concentration of the preparation was particularly high, that is, 4.82 mg ml^{-1}. Smaller relative fluorescence changes are more typical, but values of more than 100% are not unusual.

The aim of the measurements shown in Figure 7.10 was to determine the enzyme's dissociation constant, K_d, for Mg^{2+} from the Mg^{2+} concentration dependence of the observed rate constants, k_{obs}, or reciprocal relaxation times, $1/\tau$, of the traces. Mg^{2+} is a necessary cofactor of ATP. Thus, phosphorylation of the enzyme by ATP doesn't proceed unless ATP is complexed with Mg^{2+}. Taking into account the competition for Mg^{2+} complexation by free ATP in solution in the data analysis, the enzyme's K_d for Mg^{2+} could be determined to be 0.069 (±0.010) mM. This value is indistinguishable from the K_d for complexation of Mg^{2+} by free ATP in solution in the absence of Na^+,K^+-ATPase of 0.071 (±0.003) mM, which was determined by isothermal titration

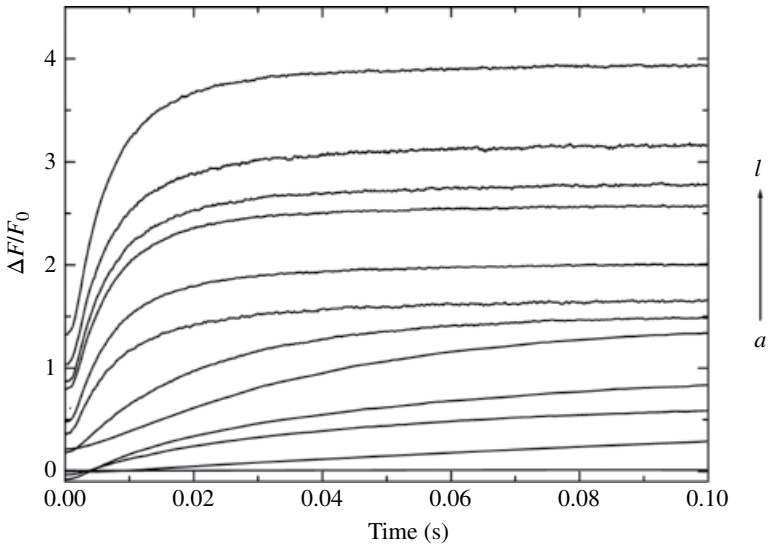

FIGURE 7.10 Stopped-flow fluorescence transients obtained on mixing Na^+,K^+-ATPase-containing membrane fragments noncovalently labeled with RH421 with ATP in the presence of 130 mM NaCl and varying concentrations of $MgCl_2$. The curves *a* to *l* represent increasing Mg^{2+} concentrations from 0 to 5.0 mM after mixing. Reprinted from Ref. 70 with permission from Elsevier.

calorimetry [71]. The similarity of these two values suggests that the enzyme itself has no significant effect on the strength of Mg^{2+} complexation and that Mg^{2+} is held within the enzyme indirectly via complexation with ATP.

This is purely one example of a stopped-flow kinetics study of the Na^+,K^+-ATPase utilizing RH421. Many others have also been published; see, for example, Refs. 50, 59, 72–84. Together with the results from other kinetic studies, sufficient information has now been accumulated to allow theoretical simulations of the entire reaction cycle of the Na^+,K^+-ATPase to be carried out under physiological conditions [85].

7.5.6 Origin of the RH421 Response

The origin of the fluorescence response of RH421 in open membrane fragments is a subject that is still the topic of current research. It is clear that RH421 responds to changes in local electric field strength, but what is the origin of the changes in local field strength?

Let us consider first the ATP mixing experiment that produces the large increases in RH421 fluorescence shown in Figure 7.10. Using chymotrypsin-modified Na^+, K^+-ATPase, which is still able to be phosphorylated but is no longer able to transport Na^+ ions, Stürmer et al. [86] found that the fluorescence changes of RH421 previously induced by the addition of ATP to native Na^+,K^+-ATPase were abolished, thus indicating that the phosphorylation reaction alone is insufficient to produce a fluorescence change. Furthermore, Pratap and Robinson [72] found that after treatment with oligomycin, an inhibitor that has no effect on the rate of phosphorylation and is

thought to act by blocking the conversion of E1P to E2P, the rate of the fluorescence change of RH421-labeled Na^+,K^+-ATPase membrane fragments induced by mixing with ATP decreased by two orders of magnitude. In a similar fashion, Cornelius [76] found that the addition of ADP dramatically slowed the kinetics of the RH421 fluorescence change. This indicates that ADP increases the back reaction rate of a step preceding the step responsible for the fluorescence change. It is known that ADP can stimulate the dephosphorylation of the E1P state (see Fig. 7.9). All of these results are consistent with the conclusion that the RH421 fluorescence change is due to either the deocclusion of Na^+, which occurs simultaneously with the conformational transition $E1P(Na^+)_3 \rightarrow E2PNa^+_3$, or the subsequent release of Na^+ from $E2PNa^+_3$ to the external solution. The term "deocclusion" refers to an opening of the enzyme to expose ion binding sites to the adjacent aqueous solution. "Occlusion," on the other hand, is a closing of the enzyme so that ions become trapped within the protein matrix with no access to aqueous solutions on either side of the membrane.

Over the last few years, crystal structures of the Na^+,K^+-ATPase have been determined by X-ray crystallography [87–91]. Based on these structures, it is now possible to theoretically calculate the electric field strength changes in the adjacent membrane when Na^+ or K^+ ions are released to or bind from, respectively, the extracellular solution. The results of these calculations [92] indicate that it is very unlikely that RH421 directly senses the electric field strength changes that arise in the membrane due to the release or binding of Na^+ or K^+ to or from the extracellular solution. The fields are expected to be effectively screened by the intervening mass of protein. In comparison, experimental data supporting the conclusion that the dye detects occlusion or deocclusion reactions has recently been obtained [92]. A useful tool for discriminating binding and occlusion is the cation benzyltriethylammonium (BTEA), which is able to bind to the transport sites of the E2P state but which can't be occluded because of its bulky size. It has been found that the addition of K^+, Rb^+, or Cs^+ ions, which are all known to be capable of being occluded, to phosphorylated Na^+,K^+-ATPase-containing membrane fragments causes a significant drop in RH421 fluorescence. This is in stark contrast to BTEA, which causes only a very small increase in fluorescence. These results, therefore, imply that the large drop in RH421 fluorescence when ions interact with the E2P state and the large increase in fluorescence when the enzyme is phosphorylated in the presence of Na^+ ions are due to changes in the state of ion occlusion, not in the state of ion binding per se.

The mechanism by which ion occlusion might cause an RH421 fluorescence change still requires further investigation. However, it was suggested by Frank et al. [58] that a protein conformational change, such as occlusion, might cause a reorganization of the lipids surrounding the protein. This appears to be a feasible origin for the RH421 response because it is known from fluorescence measurements [93] on pure lipid vesicles that the excitation spectrum of RH421 is sensitive to lipid packing, which influences the magnitude of the lipid electrical dipole potential. The effect of lipid packing on dipole potential could be a direct consequence of the change in packing density of the lipid dipoles and associated water dipoles within the membrane/water interface but could also arise partially from a change in water penetration into the membrane, producing a change in the local dielectric constant (to which the electric field strength is inversely proportional). Therefore, any reorganization of the

lipids surrounding the protein occurring as the result of a protein conformational change could well change the electric field within the membrane and hence yield an RH421 fluorescence response. Further support for such a mechanism comes from low resolution X-ray crystallographic measurements [94] on the related P-type ATPase, the sarcoplasmic reticulum Ca^{2+}-ATPase, which indicate that conformational changes of the protein are indeed accompanied by local deformations of the surrounding lipid bilayer. Further investigations into the origin of the RH421 response could, therefore, yield valuable information of fundamental scientific importance concerning the nature of lipid–protein interactions.

7.6 CONCLUSIONS

The utilization of stopped-flow fluorimetry together with voltage-sensitive fluorescent probes is a powerful combination of techniques that has already yielded much valuable information on the kinetics and mechanism of the Na^+,K^+-ATPase. However, these are not standardized research procedures that will produce immediate results of publishable quality. To obtain the best quality data, one needs to be aware of the pitfalls, for example, protein inhibition by the probe, inhibition by the probe of its own response, and the appropriate choice of excitation wavelength. To this point in time, the methods described in this chapter have only been applied to ion pump research in a limited number of laboratories. It is hoped that the tips provided here will help in extending their use and that more valuable information on a wider range of membrane proteins will be obtained. A further factor that may have limited the application of fast voltage-sensitive dyes is the lack of a solid understanding of the origin of their fluorescence response. However, rather than a disincentive, the authors feel that this should really be a stimulus for further research. The resolution of the issue of the dyes' response is likely to lead to information of broad importance to the understanding, not just of a particular ion pump, but how lipids and proteins interact with one another in membranes in general.

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