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Continuous monitoring of enzymatic activity within native electrophoresis gels: Application to mitochondrial oxidative phosphorylation complexes

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Abstract

Native gel electrophoresis allows the separation of very small amounts of protein complexes while retaining aspects of their activity. In-gel enzymatic assays are usually performed by using reaction-dependent deposition of chromophores or light scattering precipitates quantified at fixed time points after gel removal and fixation, limiting the ability to analyze enzyme reaction kinetics. Herein, we describe a custom reaction chamber with reaction media recirculation and filtering and an imaging system that permits the continuous monitoring of in-gel enzymatic activity even in the presence of turbidity. Images were continuously collected using time-lapse high resolution digital imaging, and processing routines were developed to obtain kinetic traces of the in-gel activities and analyze reaction time courses. This system also permitted the evaluation of enzymatic activity topology within the protein bands of the gel. This approach was used to analyze the reaction kinetics of two mitochondrial complexes in native gels. Complex IV kinetics showed a short initial linear phase where catalytic rates could be calculated, whereas Complex V activity revealed a significant lag phase followed by two linear phases. The utility of monitoring the entire kinetic behavior of these reactions in native gels, as well as the general application of this approach, is discussed.

Keywords

blue native electrophoresis; in-gel activity; mitochondria; oxidative phosphorylation

INTRODUCTION

Separation of protein complexes in polyacrylamide gels under native conditions [1] has various important applications [2]. Some of these applications include: determination of oligomeric states [3], the identification of physiologically relevant protein interactions [4], and the detection of protein assembly deficiencies [5]. A unique aspect of native gel electrophoresis is the visualization of “native” enzymatic activity in a small volume within the gel, requiring only micrograms of protein. This contrasts with traditional solution enzymatic activity assays with relatively large amounts of dissociated protein that require organelle and subsequent protein purification in the presence (and with possible

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interference) of excess detergents to maintain hydrophobic complexes in solution. In the widely used blue native electrophoresis [1;2], protein complexes are mixed with Coomassie blue dye, which readily binds to hydrophobic sites and confers a negative charge even to basic proteins, allowing them to migrate to the anode at a pH close to neutral. Furthermore, the negative charge at the surface of proteins greatly reduces aggregation, which is especially a problem in the case of membrane proteins. When Coomassie blue is found to interfere with a particular enzymatic reaction, the dye can be substituted by mixtures of anionic and neutral detergents to impose a negative charge while maintaining solubility of hydrophobic proteins resulting in a clear native gel [6-9]. Methods have also been developed with a colorless Coomassie analog, termed a ghost native gel, although the resolving ability of this analog is not as good as Coomassie blue [10].

Native gel electrophoresis was originally developed to study mitochondrial oxidative phosphorylation complexes (MOPCs), which are multi-subunit enzymes inserted in the inner membrane of mitochondria. MOPCs participate in the synthesis of ATP (at Complex V) by transferring electrons from substrates such as NADH (oxidized by Complex I) and succinate (oxidized by Complex II) via ubiquinone and cytochrome *c* (oxidized and reduced, respectively, by Complex III) to oxygen (reduced by Complex IV), and by coupling these redox reactions to the generation of an electrochemical proton gradient (at Complexes I, III and IV) [11]. MOPCs can be easily separated from one another and visualized by native gel PAGE using micrograms of protein obtained from tissue homogenates or biopsies without the need of purification or mitochondrial isolation [2;6;12;13]. This powerful technique has been complemented by the development of in-gel assays that allow the determination of MOPCs activities [9;13;14]. These assays rely on the formation of insoluble precipitates in the protein band where a particular MOPC catalyzes a reaction that results in the deposition of the precipitate. The in-gel reaction kinetics of Complex IV and Complex V were evaluated in the new imaging system reported in the present work.

The oxidative polymerization of diaminobenzidine was originally developed to determine the orientation of the cytochrome *c* binding site in Complex IV on microscopic images [15]. Diaminobenzidine is directly oxidized to form the insoluble polymer by cytochrome *c*, which is in turn oxidized by Complex IV and available to accept electrons from more diaminobenzidine molecules. There is evidence that this reaction is catalytic and not simply quantitative (i.e. reports multiple turnovers and not just a few) based on the sensitivity of this assay to cyanide and azide [15], which indicates that oxygen consumption is required to form an observable amount of indamine precipitate. Therefore, the in-gel activity assay of Complex IV should report on a significant fraction of the reaction elements.

The Complex V assay, which directly monitors the release of phosphate from ATP hydrolysis by formation of insoluble lead or calcium phosphate [16;17], reports the reverse of the ATP synthetic reaction that supplies the majority of ATP to most cells. In the presence of detergent, the reverse reaction is the only possible assay since no membrane potential is present to support ATP synthesis. However, both the synthetic and ATPase reaction require the rotation of the subunit, one of the most intricate actions of the enzyme complex. It is also important to point out that the kinetics of Complex V ATPase activity in solution is non-linear and complicated by many interacting factors [18]. This reaction is sensitive to oligomycin at least under some native gel conditions [9;19], suggesting that multiple turnovers take place, given that this inhibitor blocks the rotational catalysis of the F₁ sector of Complex V relative to its F₀ hydrophobic portion [20]. The Complex V in-gel assay has been extensively used to study the relative activities of monomeric and oligomeric forms of the enzyme [19;21;22].

In most cases, in-gel enzymatic assays have been performed by collecting images of the activity gels at the end of a predetermined time period (usually of several hours) when the protein band of interest has become saturated with the corresponding precipitate. The few studies that have attempted to obtain time curves [19] have had to wash and fix separate gels or lanes to collect images at different time points due to the high turbidity formed in the assay medium where the gels are submerged, requiring the use of more protein and introducing an additional source of variability. Image analysis has also been reduced to simple densitometric quantification of these overly saturated protein bands, which hinders the possibility of analyzing the kinetic behavior of in-gel activities in a consistent and reliable manner.

Herein, we describe a system that permits the continuous collection of MOPC in-gel activities using time-lapse photography. This was accomplished in a custom built mixing chamber with gels secured to the bottom and reaction media constantly circulated over the gel. Any by-products that cloud or interfere with the optical observation of the reaction products in the gel were removed with a high capacity filtering system. We also characterize an image analysis routine that corrects for background precipitate deposition and color changes that in addition generates kinetic traces with a time resolution only limited by the rate of image acquisition. These initial studies illustrate the utility of the system but also the non-linear reaction kinetics of Complex V within the gel system.

MATERIALS AND METHODS

Materials

Cytochrome *c* from bovine heart, 3,3'-diaminobenzidine, ATP disodium salt, $\text{Pb}(\text{NO}_3)_2$, glycine, and Na_2SO_3 were purchased from Sigma Chemical. N-dodecyl- β -D-maltoside (dodecyl maltoside) was from Calbiochem, and MgSO_4 was from Mallinkrodt. NativePAGE™ Novex 4-16% Bis-Tris Gels, Sample Buffer, Running Buffer, Cathode Buffer and 5% G-250 sample additive were purchased from Invitrogen. All other reagents were of analytical grade and purchased from available providers.

Sample preparation and Native PAGE

All procedures performed were in accordance with the Animal Care and Welfare Act (7 U.S.C. 2142 § 13) and approved by the NHLBI Animal Care and Use Committee. Hearts were harvested from anesthetized pigs after injection of KCl to induce arrest and perfused *in situ* with cold buffer A (0.28M sucrose, 10mM HEPES, 1mM EDTA, 1mM EGTA pH 7.1) to prevent warm ischemia and remove blood and extracellular Ca^{2+} as previously described [23]. Approximately 3 g of left ventricular free wall was dissected of all fat and connective tissue on ice, and minced with scissors in 15 ml of cold buffer A. This suspension was homogenized for 10 s in a 50 ml tube at 40% power using a tissue homogenizer (Virtis, Gardiner, NY). The rest of the free ventricular wall (~80 g) was processed as described previously to isolate mitochondria [24]. One modification was that 1 mM K_2PO_4 was added to buffer A for the first mitochondrial re-suspension to avoid phosphate depletion of the mitochondrial matrix [25]. Mitochondria were then washed twice with buffer A alone, once with buffer B (137mM KCl, 10mM HEPES, 2.5mM MgCl_2 , 0.5mM K_2EDTA), and finally re-suspended in buffer B.

Alternatively, hearts were obtained from rabbits and Langendorff-perfused as previously reported [26]. Briefly, hearts were removed from the anesthetized animal and placed in ice cold saline for transfer to the perfusion apparatus. The hearts were simultaneously perfused at 37°C at a constant pressure of 100 mm H_2O with a coronary flow of ~50 ml/min. The perfusion medium was composed of 115 mM NaCl, 4 mM KCl, 1.6 mM CaCl_2 , 1.4 mM

MgSO₄, 1 mM KHPO₄, 25 mM NaHCO₃, 5.6 mM glucose, and 3 mM Na- L-lactate. The solution was continuously equilibrated with 95% O₂ and 5% CO₂ to maintain the pH at 7.4. Heart rate, perfusion pressure and developed pressure were monitored continuously. After 40 min of perfusion, one heart was arrested for 5 min by addition of KCl, while a second heart was titrated with dobutamine to obtain a ~2-fold increase in heart rate and maintained at that rate for 5 min. Both hearts were terminally perfused with ice cold saline prior to dissection of 2 g of left ventricular free wall, which was homogenized in 20 ml of cold buffer A.

Complex IV content in the heart homogenates and in the mitochondrial suspension was determined spectrophotometrically as previously described [23;27] using Triton X-100 solubilization followed by reduction with ascorbate in the presence of cyanide for tissue homogenates and dithionite in the case of mitochondria. Approximately 0.1 ml of each sample was dissolved in 1 ml of 2% (vol/vol) Triton X-100 in 0.1 M potassium-phosphate buffer, at pH 7.0. After mixing, the suspension was centrifuged for 1.5 min at 13,000g to remove any residual connective tissue and solid material. The difference in absorbance at 605 nm between the reduced and oxidized forms of the enzyme, after baseline correction, was used to calculate the cytochrome *aa₃* content using extinction coefficients of 10.8 and 12 mM⁻¹ cm⁻¹ for tissue homogenate (due to the presence of myoglobin) and mitochondria, respectively.

Blue Native PAGE was performed according to the protocol suggested by Invitrogen protocol for the Bis-Tris Novex system. The standard gel system using 4-16% gradient pre-cast gels available from Invitrogen was used for all of the native gels used in this study. An amount of tissue homogenate or mitochondrial suspension equivalent to 1.25 nmol of Complex IV was centrifuged at 10,000g for 5 min, and the pellets were resuspended in 0.5 ml of NativePAGE Sample Buffer. 0.125 ml of dodecyl maltoside 10% was added to solubilize the mitochondrial proteins. After mixing and centrifuging at 13,000g to remove insoluble material, 6.3 l of 5% G-250 Sample Additive was added to 0.1 ml of the supernatants. Between 3 and 30 l (corresponding to 6-60 pmol of Complex IV) were loaded in 8 of the 10 wells of the gel (the two outer lanes were not used because protein bands were distorted in them). Dodecyl maltoside to a final concentration of 0.02% was added to the cathode buffer, and electrophoresis was performed at 150 V for 1 h, followed by 250 V for 1.3 h at 4°C. For Complex IV activity assays, the dark blue cathode buffer was replaced with light blue buffer (containing 10 times less cathode buffer additive than the dark blue) to decrease the staining of the gel. High-resolution Clear Native PAGE was performed as described before [9] using 0.02% dodecyl maltoside and 0.02% deoxycholate in the cathode buffer and omitting the addition of Coomassie blue and G-250 Sample Additive.

Reaction chamber and image acquisition

The reaction incubation chamber for these studies (shown in Fig. 1) was a custom constructed 10×4.5×2 inch Lucite rectangular bath with interchangeable bottom sheets for generating contrast for different assay conditions. No effort to minimize the reaction medium volume was attempted in this study, although both chamber size and depth could be reduced to minimize the volume of reaction media required. This would be especially convenient when high-cost reagents are used, such as cytochrome *c* (for Complex IV assays) or ATP (for Complex V assays). Space can be minimized if the region of the gel where the bands of interest are located is cut and placed in a smaller reaction chamber. In the present study, the entire gels were secured within the chamber either by simply removing the top plastic cover from the electrophoresis cartridge, to permit access of the reaction medium, and placing the gel/cartridge half on the bottom of the chamber secured in place with small plastic weights placed on the edges well away from the protein loaded lanes of the gel. For some experiments the entire gel was removed from the electrophoresis cartridges and pinned

down on a translucent silicone slab on the bottom of the reaction chamber. This latter approach permitted cutting regions of gels from different electrophoresis or pre-incubation conditions and pinning them on to the silicone slab in the same assay run. This also allowed the direct comparison of different gels reaction kinetics under identical conditions. The integrity of the precast commercial gels (1.0 mm of thickness) is well maintained through this manipulation and subsequent mixing within the chamber. Thinner gels may be cast if desired to increase the accessibility of substrates to all protein loaded. However, care should be taken to decrease the flow rate and mixing speeds so as to avoid tearing these more fragile gels, especially in the top region of the gel where acrylamide concentration is lower.

For monitoring in-gel reactions two basic incubation systems were used. For reactions where color was developed in the gel with no interference in the bath, such as Complex IV, the only mixing was provided by two magnetic mixers on opposite sides of the long axis of the chamber (see Fig. 1). For those assays where the bath became turbid during the progress of the reaction, such as Complex V, a perfusion system was used to constantly filter the medium along with the magnetic mixers. For continuous filtering, input and output fluid ports on opposite ends of the long axis of the chamber were connected by tubing to a Cole-Palmer Master Flex peristaltic pump and to two MiniKap (Spectrum Laboratories, Rancho Dominguez, CA) filters connected in parallel, each with 500 cm² of 0.2 μ m pore size membrane (shown in Fig. 1). The flow rate of this system was approximately 100 ml/min, and the direction of flow was set so that the filtered assay solution reached the higher molecular weight bands (including those of Complex V) first as it entered the reaction chamber. We also found that the direction of the flow should be set so that the unfiltered media enters through the end of the MiniKap filters in which the label is found in order to avoid clogging, and that the filters should be connected between the outlet of the pump and the reaction chamber (see Fig. 1). For the Complex V assay, this filtering system allowed the solution to remain in essence optically clear throughout the experimental time course. It should be noted that the ~1000 cm² of filter area was required to prevent significant back pressure on the pump as the media was filtered during the development of the reaction. If temperature control is desired, a water bath can be used to immerse the filters and part of the tubing. The chamber itself can be warmed using the temperature control of the magnetic stirrers. Colder temperatures can be maintained by immersing the bottom of the reaction chamber in a larger vessel with water connected to a refrigerated bath.

The same imaging protocol was used during both the non-perfused (Complex IV) and perfused (Complex V) protocols. The two fiber optic illuminators of an unfiltered white light source (Cole-Palmer optic fiber illuminator) were placed a few cm above the container to illuminate the gel at a shallow angle with respect to the surface of the assay solution so as to avoid as much reflected light as possible (see Fig. 1). A cover sheet of glass or plastic over the top of the chamber was found to trap bubbles and increase interference from scattered light. Thus, imaging was conducted directly over the reaction media with some interference due to ripples formed on the surface. Polarizing lenses on the camera did not improve scattering interference significantly and were not used. All other lights in the room were eliminated for assays dependent on scattered light, such as Complex V. As shown in Fig. 1, a Nikon D700 digital camera with a 105mm Nikon Macro lens was secured ~25 cm above the surface of the gel, and connected via a USB port to a laptop computer running a camera control software program for time lapse photography (Nikon Camera Control Pro 2 (version 2.9)). The camera focus and exposure were manually set. In general, a shutter speed of 1/30 s and an aperture set between f/8 and f/4 were used depending on the assay. Time lapse photography with this system can be set as fast as 1 frame/s, limited by digital frame transfers. Typical experiments were collected with a frame rate of 5-20 s depending on the total collection time, which ranged from 1.5 to 3 h, yielding datasets of 400-600 images. In principle much faster image framing rates, or even video frame rate (30 frames/sec), could

be used in this system for very fast reactions. We found the high spatial resolution of this camera (2128x1416 pixels/frame) and a framing rate of 5-20s were more than adequate to define the kinetic reactions evaluated in this study of MOPCs activity. Each image was saved on the PC as a JPEG RGB file which basically provided a simultaneous red, green and blue filtered image of the reaction sequence. The highest contrast and signal to noise color channel was used for analysis (see below). Windows Movie Maker (Microsoft) and Virtual Dub programs were used to create video AVI or WMV files from the image series for qualitative visualization.

Complex IV and V assay conditions—The main Complex IV band migrated just on top of a ridge of the back plastic plate of the pre-cast gel, which reflected light upon illumination. Therefore, the gel was separated from both standard plastic plates and pinned down to the translucent silicone slab on top of a white surface. The container was filled with 400 ml of Tris 50 mM (pH 7.4) in which 0.2 g of diaminobenzidine had been dissolved to obtain a final concentration of 1.4 mM. To begin the reaction, 2 ml of water containing 40 mg of bovine heart cytochrome *c* were added to the assay solution, resulting in a concentration of 8 M. The addition of catalase to the assay, which has been used in other studies [17;28], was omitted in the present work considering that it has been reported to have little effect [9;15], and that no generation of hydrogen peroxide should be expected in a blue native gel where the respiratory chain is disrupted by protein separation.

For monitoring Complex V, only the upper plastic plate was separated from the gel and discarded, and the gel still attached to the lower plate was gently shaken for 30 min at room temperature in 20 ml pre-incubation buffer containing 35 mM Tris (pH 7.8), 270 mM glycine and 14 mM MgSO₄. The gel was placed face up in the imaging container, but over a black surface to enhance contrast for this assay. In some circumstances, when different pre-incubation or gels were desired to be directly compared, the lower plastic plate on the gel system was also removed before pre-incubation and the regions of the gels pinned to a silicone slab for direct comparisons. The reaction medium was prepared by adding 1.9 ml of KOH 4 M to 400 ml of pre-incubation buffer to maintain the pH at 7.8 before simultaneously adding 0.8 g of Pb(NO₃)₂ and 1.76 g of ATP disodium salt, yielding a final concentration of 6 mM and 8 mM of lead and ATP, respectively. The solution was vigorously shaken for a few seconds and then filtered by vacuum using a Nalgene MF75 filter unit with 0.2 μm pore size cellulose nitrate membrane. This solution was poured in the reaction chamber where the gel was placed, bubbles were gently removed, and the two plastic weights were placed on each side of the gel. The two magnetic stirrers on each end of the container were started at the same time as the peristaltic pump, set to 25% of maximal flow, which corresponded to ~100 ml/min. The direction of the flow was such that the top portion of the gel was facing the incoming filtrate.

Reactions reported in the present work were performed at room temperature (22°C). Higher temperatures (up to 37°C) resulted in faster non-enzymatic rates of precipitate formation especially in the Complex V assay (data not shown), which could still be corrected by the imaging analysis if the pump flow rate was increased to 150 ml/min. Enzymatic rates were even higher as the temperature was increased. However, linear phases were much shorter in the Complex IV assay and lag phases in Complex V kinetics were less discernible (see below). Lower temperatures (down to 10°C) proved useful when analyzing kinetics in clear native gels, but prolonged the lag phases in Complex V kinetics (not shown), which made it difficult to identify of the linear phase where initial rates can be calculated.

Image analysis

Data analysis scripts were written in the IDL program version 7.1 (Exelis Visual Information Solutions). All analysis was performed directly on the full resolution JPEG RGB files directly transferred to the computer during the acquisition. For Complex IV, the formation of the indamines precipitate on the enzyme changed the band color from light blue to dark brown. Based on signal to noise ratios, we selected the blue channel for analysis and inverted the image intensity so that the pixel signal increased as the reaction progressed to facilitate the interpretation of the data. A higher intensity and a better signal to noise ratio was observed when the inverted images were analyzed in the blue channel compared to the green, although the linear phase was shorter and saturation was achieved earlier in the blue. The slopes calculated from the linear phases were approximately 3-5 times higher in the blue, and in the green the slope at the lowest protein amount (3 pmoles of Complex IV) was difficult to calculate due to a very low intensity. For Complex V, the integrated intensity produced by the white phosphate precipitate in each protein band was slightly lower (~30%) in the red channel than in the green and blue, and the signal to noise ratio was similar in the red and green and approximately 3-fold better than in the blue, where saturation was reached earlier. Therefore, the green channel was selected and was used without inversion given that pixel intensity increased as the white precipitate formed.

Image analysis was performed using two approaches. Most of the analyses were conducted with regions of interest (ROI) that were selected around the entire region of a MOPC band based on the last image of the time course to integrate total intensity. This is analogous to most densitometric measurements. Control regions in the gel, of the same pixel dimensions and well outside of the protein bands were selected as controls (cROI). Time courses for the intensity of each ROI over time were created for kinetic analyses. The cROI integrated intensity was subtracted from its corresponding MOPC band to correct for global changes in the background and light-source. When appropriate, linear regions in each of these averaged and corrected kinetic traces were manually selected and initial rates were calculated by linear regression.

In some cases the time course of each pixel within an ROI was analyzed to evaluate the topology of the enzymatic reaction within a protein band on the gel. In this analysis, the time course was divided into three equal time points and linear regressions applied to each individual pixel of the ROI over time. The slopes of these reactions were extracted and assigned to the pixel position to generate three maps of enzymatic activity at the beginning, middle and end of the reaction time course. The topology of these reactions was analyzed by creating images of the slope data after scaling for image presentation. The scaling was done using a resident IDL routine, "bytscl", where each pixel of the ROI, P_x , was assigned a value relative to the slope, x , using the following normalization scheme: $P_x = (255 + 0.9999) \times (x - Min)/(Max - Min)$ where *Min* and *Max* refer to the maximum and minimum slope value in the ROI. This normalization was conducted on each image individually to highlight the topology of slopes within a time band. To our knowledge, these are the first evaluations of the topology of reactions rates within native gels. Absolute, non-normalized, histograms were also generated for each selected time range to represent the distribution of pixels with different absolute precipitate formation rates.

RESULTS AND DISCUSSION

Complex IV in-gel assay kinetics

The image acquisition and analysis described in the present work was applied to obtain the kinetic traces shown in Fig. 2A for the Complex IV in-gel activity assayed for 1 h 40 min with a time resolution of 20 s at protein amounts ranging from 6 to 60 pmoles of enzyme. No temporal filtering was applied to these data, or any subsequent traces, to reveal the actual

signal to noise ratio generated by the methodology. Clearly, high pass filtering would be appropriate and improve the data analysis. No lag period in the reaction kinetics of the enzyme were detected, indicating that time for substrate diffusion into the gel was not required for the start of the reaction. Although several faint bands showing activity were detected in each lane (see Supplementary material), only the strongest band was used for the present analysis. As shown in Fig. 2B, the slopes calculated from the linear phases (below 1000 s) depended linearly on enzyme concentration up to 50 pmol of protein with an excellent correlation coefficient ($R = 0.996$). Detector saturation was evidently reached below the most abundant protein band (corresponding to 60 pmoles), resulting in an abrupt loss of linearity. The 60 pmoles data was not used in the linear correlation, given that saturation had evidently been reached at such a high protein concentration. An important limitation of native gel assays is the difficulty in calculating specific activities from the formation of precipitates. Arbitrary units are used to describe pixel intensity because an actual quantification of light absorbed or scattered would depend on illumination intensity and camera sensitivity. The normalization of activity in a gel using an in-solution assay with known quantities of enzyme would also be dubious because proteins are not expected to show the same rates when concentrated in a protein band with Coomassie dye bound relative to an aqueous media at very high dilution and in the absence of dye. Therefore, in-gel assays are relevant only for relative comparisons of enzymatic activity, but cannot be easily correlated to specific rate units of catalysis.

The observation that the reaction reached saturation at a different level of signal intensity depending on the concentration of protein (see Fig. 2A) seems to disagree with the assumption that the in-gel assay reports multi-turnover catalysis of Complex IV. That is, it might be expected that at all protein concentrations the same maximum intensity value should be approached at infinite time. However, this is apparently not the case with the low protein bands, which “saturated” well below the maximum reached at higher protein concentration bands. These results suggest that the protein was inactivating around 1500 sec in the gel, or that some other image artifact was occurring. A possible artifact involved “blooming” of the protein band at higher protein concentrations; that is, the protein band is more diffuse with higher protein loading. In an ROI integration procedure, a given amount of protein spread over a large volume within the ROI will result in a larger integrated signal than if the protein is concentrated in a small region where saturation of the detection system occurs rapidly. Another way to look at this is that if the protein is differentially dispersed within the ROI, each region of the ROI will have a different deposition rate. As long as none of the regions reach saturation, the overall integration will provide a good measure of the average ROI enzyme activity, allowing appropriate kinetic analysis.

To address this “blooming” issue, rates of precipitate deposition were obtained for each pixel in the protein band. As shown in Fig. 3 for the 60 pmol protein band, the central region was responsible for the highest rates of indamine formation, reaching saturation within the first third of the time course. Surrounding regions where protein was less abundant contributed to the much slower and less intense precipitate deposition during the remainder of the reaction time. This heterogeneity is very evident in a histogram plot, which shows that even in the first third of the time course different pixel groups cluster at different rate values, whereas in the second and last thirds the slower reaction results in a compression of the rate distribution at only lower values. The rapid saturation of the central core of the protein band therefore contributes to the apparent differential saturation observed with protein concentration in the full ROI time course data in Figure 2, which corresponds to the total amplitude (or absolute pixel intensity) shown in the inset of Fig. 3. By avoiding time points where saturation within any region of the ROI has occurred, accurate rate data proportional to the average enzyme concentration can be extracted. This temporal and multi ROI analysis assures data is analyzed before any saturation occurs, providing a better characterization of

the kinetics of the assay as well as the true kinetic behavior of the enzyme within the protein band. Regions where saturation is reached earlier probably correspond to those where a higher amount of enzyme is exposed to the surface of the gel. However, the topology analysis cannot directly distinguish between activities at different gel depths within the protein band. Nevertheless, it seems reasonable to conclude that regions with lower rates and later saturation times have less accessibility to the reaction substrates.

Complex V in-gel assay kinetics—In contrast to the Complex IV assay, which did not require filtering during the image collection and was corrected for background changes during the analysis, the activity buffer for the in-gel Complex V activity became highly turbid within the first 5 min of the reaction. The filtering system used to maintain the solution reasonably clear during the collection time (see movies in Supplementary material), and any slight changes in medium transparency were successfully corrected by subtracting the integrated intensity of control ROIs adjacent to the Complex V bands. Fig. 4A shows the overall ROI intensity kinetics of in-gel ATP hydrolysis by Complex V obtained at different protein amounts. A clearly distinct lag phase was observed, especially in the bands with the lowest protein, where this phase lasted up to 2000 s. In the bands with highest protein, this lag covered the first 800 s of the assay. Thus, the linear phase where initial rates could be calculated was located between different time points at each protein concentration. For instance, in the band with less protein, the linear phase occurred above 3000 s, whereas in the four bands with the highest amount of protein, linear kinetics were restricted to 1200–2400 s. At later time points (>4000 s), the integrated intensities in the bands with more abundant protein showed a transition to another region of linearity which exhibited a lower slope. The enzymatic rates calculated from the first linear phase (Fig. 4B) were proportional to the amount of protein loaded up to 40 pmol of Complex IV (equivalent to ~20 pmol of Complex V, assuming that heart mitochondria have 0.5 Complex V per Complex IV [29]), with a $R = 0.995$. Values above 40 pmol were not used in the analysis, in view of the clear loss of a linear dependence of the rate on the concentration of enzyme at the higher protein amounts.

The multiphasic kinetic pattern of Complex V kinetics revealed by our image acquisition and analysis methods underscores the disadvantages of collecting at most a few images after stopping the reaction and washing the gel, which is the only option in the absence an efficient filtering system. This is illustrated in Figure 5, where the ratio of the integrated areas of the 40 and 6 g protein bands are plotted as a function of time. The ratio varies from roughly 1 to 40 depending on the time point, with the differential lag resulting in exaggerated changes in activity at short incubation times. Note the correct ratio of ~6.6 is only observed briefly in the time band at 670 s and then at ~8700s. This example illustrates the dependence of the Complex V assay on the time the measurement is made, which is severely influenced by the differential delay in the reaction kinetics.

As shown in Fig. 6, calculation of precipitate formation rates in individual image pixels within the 60 g protein band of Complex V revealed that, just as with Complex IV, signal saturation was almost reached at the end of the first third of the time course at the front edge where protein was more abundant. The 60 g sample was selected since it revealed the largest saturation effect for illustrative purposes. Surrounding regions of the gel, especially in the trailing region of the band, accounted for the slower rates observed in the final two thirds of the reaction time, which is consistent with a lower protein concentration in these regions. Thus, the total amplitude or total integrated pixel intensity (Fig.6, inset) is contributed mostly by the region that becomes saturated in the first third of the reaction time course. The distribution of pixels that reacted with a similar rate was much more homogeneous than with Complex IV, as observed in a histogram, with the initial velocity corresponding exactly to the rate of the front edge of the protein band. The second and third segments of the time

course also exhibited a sharp peak in the rate distribution of the individual pixels, although not as tight as in the first phase, but still resulting in a slow transition to a lower overall rate at the latest time points. As discussed above for the Complex IV topology analysis, it can be predicted that the front edge of the protein band where saturation occurs earlier contains more protein exposed at the surface of the gel, although the imaging system used lacks the focal resolution to calculate rates at different depths of a 1 mm gel.

The lag phase in Complex V in-gel cannot be simply attributed to a delay in substrate diffusion since the lag was not observed in other MOPC reactions even using larger substrate molecules (i.e. Complex IV with cytochrome *c*) and the delay was a function of the enzyme content in the gel, which is inconsistent with bulk delivery of reactants being limiting. Supporting the notion that the lag is more related to the function of the enzyme, classical ATP hydrolysis assays of Complex V in solution have also revealed a lag phase, which has been attributed to a tightly bound ADP-Mg complex in one of the three catalytic sites of enzyme [30-32]. Anions such as bicarbonate and sulfite can relieve this inhibition by acting as analogs of phosphate and displacing the inhibitory ADP-Mg [33;34]. The pre-incubation of the gel in a solution with MgSO_4 for 30 min would in principle promote the binding of this inhibitory complex if the enzyme is able to maintain at least a stoichiometric amount of ADP bound during separation by native electrophoresis. However, the time scale of the lag phase in solution activity assays is in the order of tens of seconds at the most [31], so its relation to the much more prolonged lag phase (> 800 s) we have observed in the in-gel assay is not straightforward. To address this issue, we compared the kinetics of in-gel Complex V ATP hydrolysis with or without pre-incubating the gel in the presence of sulfite. For this experiment, Complex V solubilized directly from heart tissue homogenate was used, since we noticed that the lag phase was longer and the rate slower than in enzyme obtained from isolated mitochondria (not shown). Fig. 7 shows that pre-incubation with sulfite significantly shortened the lag phase by $\sim 50\%$, and also increased the rates calculated during the linear phase by a factor of 2. These effects of sulfite are consistent with dissociation of inhibitory ADP-Mg from the enzyme as suggested by earlier studies in solubilized Complex. However, in separate studies we found that the lag phase was unaffected by long incubations of the gel with an excess of creatine kinase and creatine in addition to sulfite to dissociate and convert all bound ADP to ATP, nor was the pre-incubation of gels with ADP-Mg able to extend the lag phase and decrease the rate of Complex V from mitochondria or tissue homogenates (not shown). Therefore, sulfite must be exerting an effect on Complex V activity by another mechanism in addition to its ability to decrease inhibition by ADP-Mg. Nevertheless, the rate increases observed with sulfite demonstrate that the in-gel Complex V assay reports multi-turnover catalysis, given that oxanions do not affect (or even inhibit) the rate of unisite ATP hydrolysis [35;36]. Importantly, these sulfite studies show that alterations in the lag period can be experimentally manipulated, suggesting that this phenomenon needs to be accounted for using this assay.

Another explanation for the long lag observed in the Complex V in-gel kinetics is the slow dissociation of Coomassie blue from the enzyme molecules in the surface of the gel. It is known that, in blue native gels, the tightly-bound inhibitor oligomycin does not inhibit ATPase activity [9], probably because the dye obstructs accessibility of the inhibitor to its binding site. Given that the dye is negatively charged, it could also prevent binding of the ATP molecule to the catalytic sites in the enzyme. To explore this possibility, we obtained kinetics of Complex V in-gel activity in clear native gels. As shown in Fig. 8, the activity of ATP hydrolysis in the absence of Coomassie blue, although it was several times faster than in blue native gels (compare to Fig. 4), still showed a lag phase that was longer at lower protein concentrations. This result indicates that Coomassie blue dissociation is not responsible for the lag phase of Complex V activity. However, since the duration of the lag in clear native gels was also shorter (>200 s) than in the blue native format (>800 s), it is

possible that binding of ATP-Mg to some regulatory site in the enzyme is necessary to fully activate the enzyme, as has been observed in solution [37]. Indeed, we found that pre-incubation of the gel in ATP-Mg eliminated the lag almost completely (data not shown), even after subsequently washing out the gel to eliminate the ATP. Irrespective of the exact mechanism for the lag phase, it is noteworthy that our imaging collection technique, which in this case acquired images every 5 s, could easily resolve kinetic features that lasted only a few minutes, which would be impossible with traditional methods in which gel images are collected tens of minutes from each other at best [19].

The importance of analyzing the full range of kinetic features of the Complex V in-gel activity is illustrated by the representative experiment shown in Fig. 9. It has previously been shown that the workload of hearts both *in vivo* and *in vitro* can influence the activity of Complex V in blue native gels [13]. Thus, we used this model to evaluate the kinetics of Complex V activity in blue native gels from tissue taken during low workload (KCl-arrested), and high workload (dobutamine-infused) perfused rabbit hearts. The protocol was essentially identical to earlier studies [13] with the difference that both hearts were perfused simultaneously at the same perfusion pressure to eliminate any variance in the time associated with tissue preparation. The acute increase of workload in perfused rabbit hearts by perfusion with dobutamine resulted in a shorter lag phase in the Complex V kinetics (~1000 s) relative to the condition in which contraction was arrested with KCl (~2000 s). Even though the difference in slope once a steady state was reached was only of 30% different between the two conditions, the difference in lag resulted in a 2.5-fold difference in integrated signal intensity around 1000 s, which gradually decreased to a 1.5 ratio. Similar results were obtained in 4 trials using this paired-heart analysis. These data suggest that most of the effects of workload on the Complex V activity was due to a change in the lag phase and not the rate of reaction. Therefore, the use of fixed time points arbitrarily chosen for densitometric quantification of the gel bands is undesirable when such information-rich kinetic patterns as the ones shown by Complex V are present, because the calculation of simple initial or integrated rates results in oversimplification of the data.

Since this is the first observation of a lag in the Complex V blue native gel assay, we are still in the process of working out the molecular mechanisms associated with this delay. It is not consistent with a limitation in ATP or Pb diffusion as it can be manipulated by incubation conditions, sulfite effects, as well as by the metabolic status of the tissue it is extracted from, working versus arrested heart. Thus, it likely does reflect some temporally dependent alteration in the Complex V that is dependent on the reaction with ATP. Clearly this kinetic approach to the blue native Complex V assay will aid in the investigation of this phenomenon.

Conclusions

The time-lapse photography approach presented in this work provides new useful topological and kinetic information on enzymatic activity within native gels. Continuous acquisition of in-gel activity of MOPCs has been demonstrated for Complexes IV and V in blue and clear native gel formats. For Complex V in-gel activity, perfusion along with in-line filtration removed the turbid by-products from the media permitting continuous monitoring. With this method, kinetic traces were obtained with a time resolution on the order of seconds, allowing the localization of the appropriate linear regions of activity where catalytic rates can be accurately determined. The topology of these reactions within the native gel was also analyzed and compared with the overall integration methods, underscoring the importance of making determinations before any region of the protein deposition had reached saturation. Other kinetic features observed might have important mechanistic implications, such as the lag in ATP hydrolysis by Complex V that was resolved for the first time in native gels, which would be difficult to detect with sparse

temporal sampling. It is concluded that this kinetic approach to the analysis of enzymatic reactions in native gels can expand and improve the interpretation of these complex reactions compared to traditional low time resolution methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Imaging system for the continuous monitoring of in-gel activity

The different components used for placing, illuminating and photographing the gel are shown schematically, together with the elements that allowed the continuous filtering and mixing of the assay solution. The directionality of the flow needed to avoid clogging of the filters and to achieve a lower turbidity of the solution in the upper molecular weight (top) region of the gel is also indicated.

Figure 2. Kinetics of Complex IV activity in a blue native gel

The integrated pixel intensities of precipitate formation by the main Complex IV band in lanes loaded with the indicated amount of enzyme from pig heart mitochondria are plotted as a function of time (A). The slopes calculated from the linear region of each kinetic trace are plotted as a function of protein loaded into the gel (B), with the solid line corresponding

to the linear regression of the slopes up to 50 pmol of enzyme loaded. The resulting parameters of the linear equation as well as the correlation coefficient are also shown. Images were collected every 20s and analyzed as described in the text. See Supplementary Material for a video of the in-gel activity.

Figure 3. Kinetics of precipitate formation in individual pixels within the Complex IV protein band

The pig heart mitochondrial protein band containing Complex IV (60 pmol of loaded protein) is shown on top with pixel intensity corresponding to the rate of precipitate formation calculated by linear regression during the indicated segment of the time course, normalized to the maximal rate at each segment. Total absolute pixel intensity at the end of the run (Total Amplitude) is shown as reference. The histogram shows the distribution of pixels that reacted at a particular rate during the first (solid line), second (dashed line) or third (dotted line) segments of the time course.

Figure 4. Kinetics of Complex V activity in a blue native gel

The integrated pixel intensities of precipitate formation by Complex V bands in lanes loaded with the indicated amount of enzyme from pig heart mitochondria are plotted as a function of time (A). The slopes calculated from the linear region of each kinetic trace are plotted as a function of protein loaded into the gel (B), with the solid line corresponding to the linear

regression of the slopes up to 50 pmol of enzyme loaded. The resulting parameters of the linear equation as well as the correlation coefficient are also shown. Images were collected every 20s and analyzed as described in the text. See Supplementary Material for a video of the in-gel activity.

Figure 5. Ratio of integrated signal intensity between low and high protein bands of Complex V in a blue native gel

The kinetic traces from the 6 and 40 pmol pig heart mitochondrial protein bands from Figure 3 were divided and the resulting trace was smoothed to decrease the noise at low signal values.

Figure 6. Kinetics of precipitate formation in individual pixels within the Complex V protein band

The pig heart mitochondrial protein band containing Complex V (60 g of loaded protein) is shown on top with pixel intensity corresponding to the rate of precipitate formation calculated by linear regression during the indicated segment of the time course, normalized to the maximal rate at each segment. Total absolute pixel intensity at the end of the run (Total Amplitude) is shown as reference. The histogram shows the distribution of pixels that reacted at a particular rate during the first (solid line), second (dashed line) or third (dotted line) segments of the time course.

Figure 7. Effect of sulfite on the kinetics of Complex V activity in a blue native gel
Kinetic traces of precipitate formation in Complex V bands from pig heart homogenates corresponding to the indicated amount of mitochondrial protein were obtained after incubating the gels in the absence (A) and presence (B) of 10 mM Na_2SO_3 for 30 min.

Figure 8. Kinetics of Complex V activity in a clear native gel

Kinetic traces of precipitate formation by Complex V bands from the indicated amounts of pig heart mitochondrial protein loaded in a clear native gel (Coomassie blue substituted for deoxycholate 0.02% in the cathode buffer) are shown. Images were collected every 5 s and analyzed as described in the text. See Supplementary Material for a video of the in-gel activity.

Figure 9. Kinetics of Complex V obtained from perfused rabbit hearts in a blue native gel
 Kinetic traces of precipitate formation in Complex V obtained from KCl-arrested or dobutamine-stimulated rabbit heart corresponding to 10 g of mitochondrial protein (A) were obtained after tissue homogenization. The ratio of the integrated signal intensity at each time

point between the two protein bands is shown (B) after smoothing to decrease the noise at low signal values.