Electroactivity of proteins

and its use in biomedicine

PROTEINS (history)

The word protein comes from the Greek πρώτα ("prota"), meaning "of primary importance" and these molecules were first described and named by JJ Berzelius in 1838. However, proteins central role in living organisms was not fully appreciated until 1926, when J B Sumner showed that the enzyme urease was a protein.

The first protein to be sequenced was insulin, by F Sanger, who won the Nobel Prize in 1958. The first protein structures to be solved included hemoglobin and myoglobin, by Max Perutz and Sir John C Kendrew, respectively, in 1958. Both proteins three-dimensional structures were first determined by x-ray diffraction analysis; the structures of myoglobin and hemoglobin won the 1962 Nobel Prize in Chemistry for their discoverers.

Proteins are an important class of biological macromolecules present in all biological organisms, made up of such elements as carbon, hydrogen, nitrogen, oxygen, and sulfur. The polymers, also known as polypeptides consist of a sequence of 20 different L-α-amino acids, also referred to as residues. For chains under 40 residues the term peptide is frequently used instead of protein. To be able to perform their biological function, proteins fold into one, or more, specific spatial conformations, driven by a number of noncovalent interactions such as hydrogen bonding, ionic interactions, Van der Waals' forces and hydrophobic packing. In order to understand the functions of proteins at a molecular level, it is often necessary to determine the three dimensional structure of proteins. This is the topic of the scientific field of structural biology, that employs techniques such as X-ray crystallography and/or NMR spectroscopy, to determine the structure of proteins.

A certain number of residues is necessary to perform a particular biochemical function, and around 40-50 residues appears to be the lower limit for a functional domain size. Protein sizes range from this lower limit to several thousand residues in multi-functional or structural proteins. However, the current estimate for the average protein length is around 300 residues. Very large aggregates can be formed

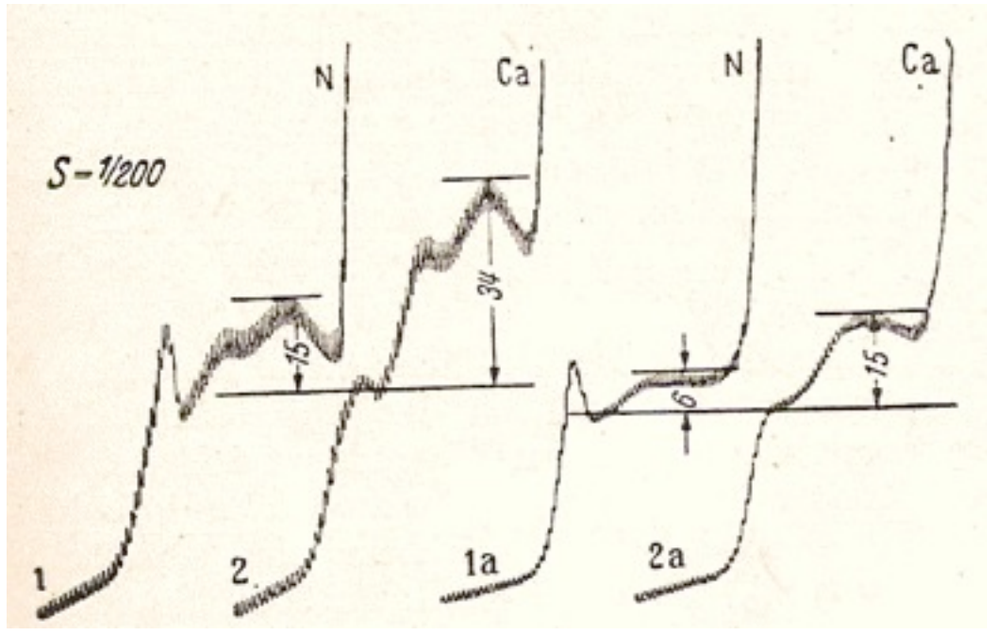
from protein subunits, for example actin - collagen and α-synuclein in Parkinson's disease

Electrochemistry showed its usefullness in the past and present analysis of DNA.

Can electrochemical analysis be equally useful in the present **protein research**?

Electrochemical analysis of proteins

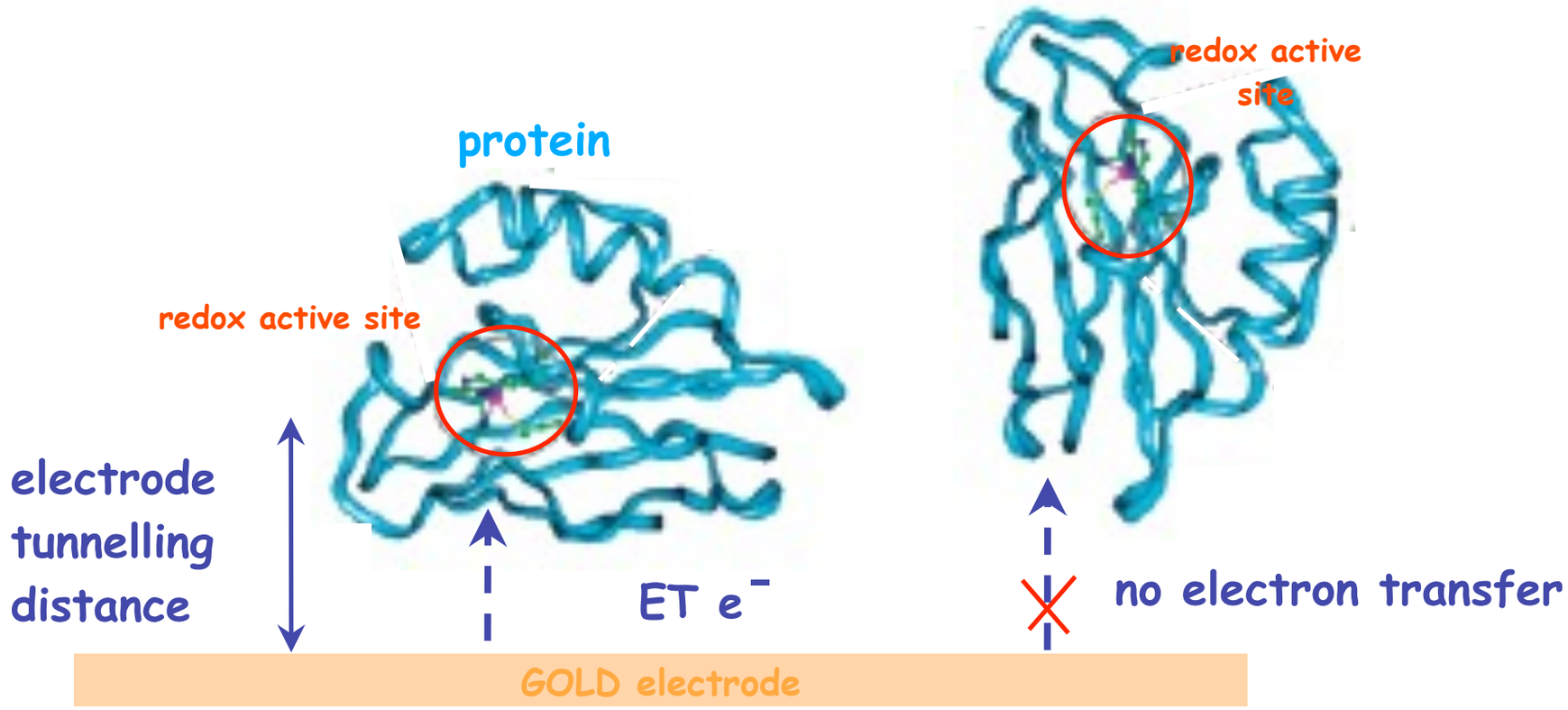
was succesfully applied in biochemistry, pharmacy, medicine and particularly in clinical oncology research for several decades in the middle of the 20th century.



Later the attention of electrochemists turned to direct electrochemistry of a limited number of redox-active center-containing proteins and the potentialities of the electrochemical methods as tools for protein analysis in molecular biology and biomedicine were neglected.

Electrochemical **responses of proteins** are dependent on the **orientation of the protein molecule at the electrode surface**

Small conjugated proteins (mostly metalloproteins) undergo fast reversible processes at electrodes

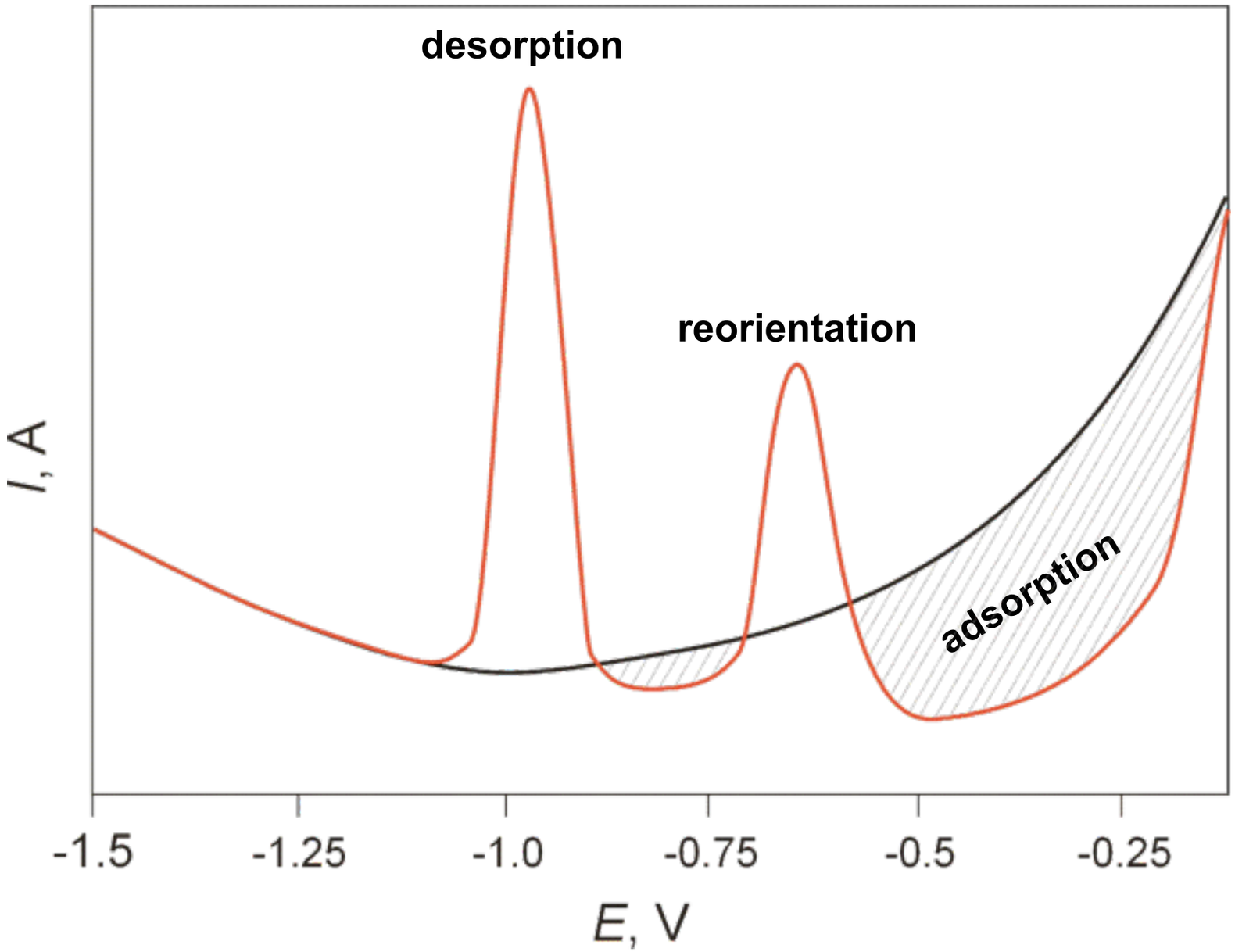


GOLD electrodes are preferable used but at these electrodes amino acid residues produce NO REDOX RESPONSES

On **CARBON** and **MERCURY** electrodes some **amino acid** residues can yield analytically useful **ELECTROCHEMICAL SIGNALS**

Phase-sensitive a.c. voltammetry or a.c. impendance measurements

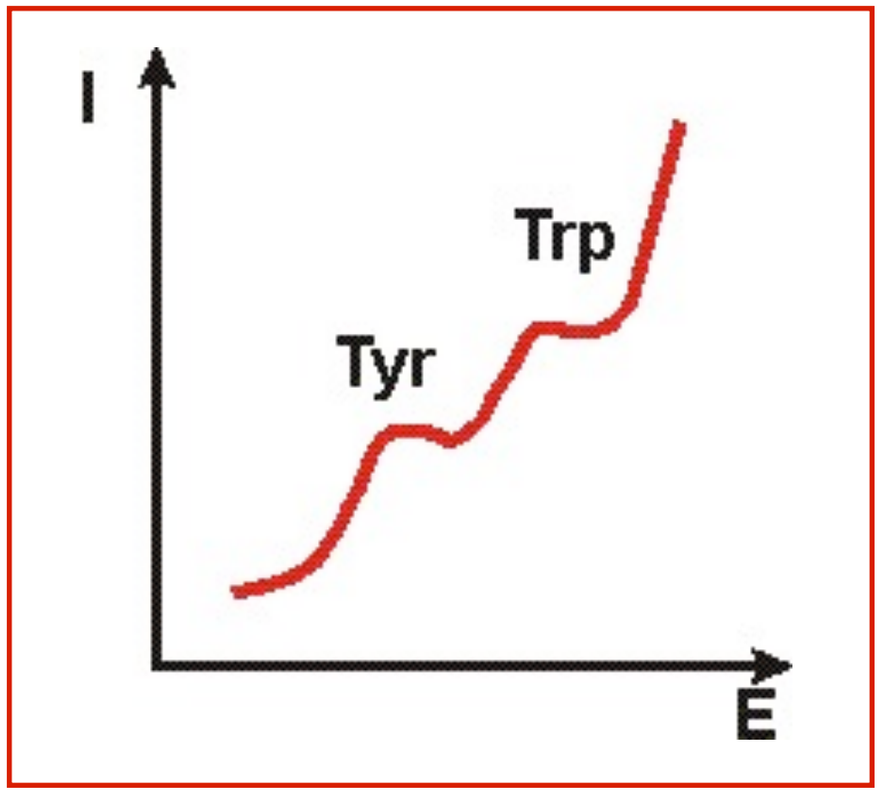
can provide information about **PROTEIN ADSORPTION/DESORPTION** behavior in dependence on the electrode potential and/or a.c. frequency



LIQUID MERCURY ELECTRODE provides very **SMOOTH SURFACE** and best **REPRODUCIBILITY**

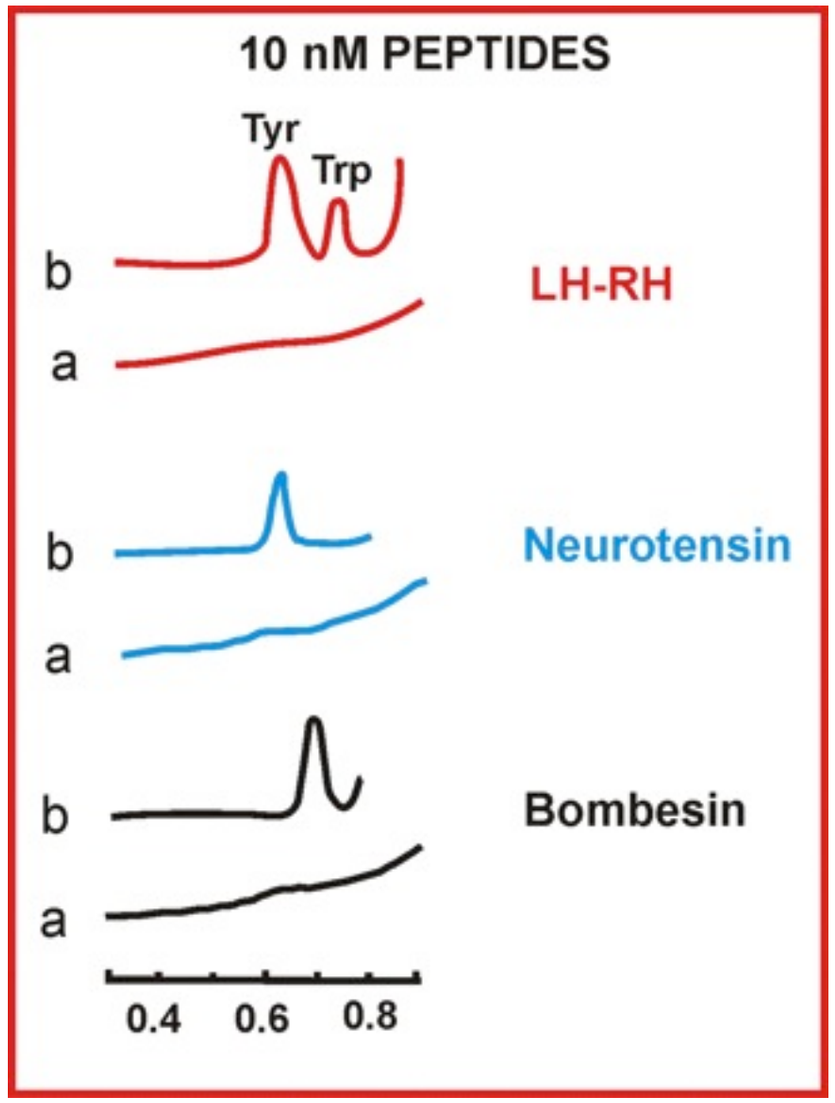
Tyr and Trp oxidation at carbon electrodes

d.c. polarography



Constant current chronopotentiometry or square wave voltammetry

1996



These techniques contain sophisticated base line correction (compensating the high background currents at carbon electrodes)

Electrochemistry has shown its usefulness in the analysis of DNA and in studies of some conjugated proteins.

Can electrochemical analysis be equally useful in the **research of all proteins**?

ELECTROACTIVITY OF AMINO ACIDS IN PROTEINS

Tyrosine and tryptophan residues are oxidized at carbon electrodes

Chronopotentimetric peak H
which is due to the ability of proteins to catalyze hydrogen evolution,
is obtained at mercury and solid amalgam electrodes

Peak H ..... because of Heyrovsky J, Hydrogen evolution, High sensitivity

Present **proteomics** requires sensitive methods for the analysis of **all** proteins. We wish to show that electroactivity of amino acid residues in proteins can be utilized in the analysis of practically all proteins, including those important in **biomedicine**.

According to F A Armstrong (Encyclop. Electrochem. Vol. 9, 2002) a problem with commonly used **metal electrodes**, such as gold, mercury, platinum and silver, is that they **lead to denaturation** and irreversible adsorption of the resulting inactive protein.

In my talk I wish to show that proteins can remain native at the bare mercury electrodes and combined with CPS peak H these electrodes can be useful in studies of changes in protein structures, incl. denaturation

Peak H differs from the previously described polarographic and voltammetric electrocatalytic signals of proteins

(i) by its ability to detect peptides and proteins down to nanomolar and subnanomolar concentrations and

(ii) by its remarkable sensitivity to local and global changes in protein structures.

Native

Weaker adsorption at Hg surface

hydrophobic groups (and some thiol and disulfide groups) burried.

Electroactive groups less accessible

Ostatná V., et al.. (2006): Native and denatured bovine serum albumin. D.c. polarography, stripping voltammetry and constant current chronopotentiometry. J. Electroanal. Chem. 593, 172-178.

native

Denatured

Stronger adsorption at Hg surface

hydrophobic groups, thiol, disulfide and other groups better accessible

Greatly increased electroactivity

Ostatná V., Palecek E. (2008): Native, denatured and reduced BSA. Enhancement of chronopotentiometric peak H by guanidinium chloride. Electrochim. Acta, 53, 4014-4021

Ostatna V. et al. (2008) Constant current chronopotentiometry and voltammetry of native and denatured serum albumin at mercury and carbon electrodes, Electroanalysis 20, 1406-1413

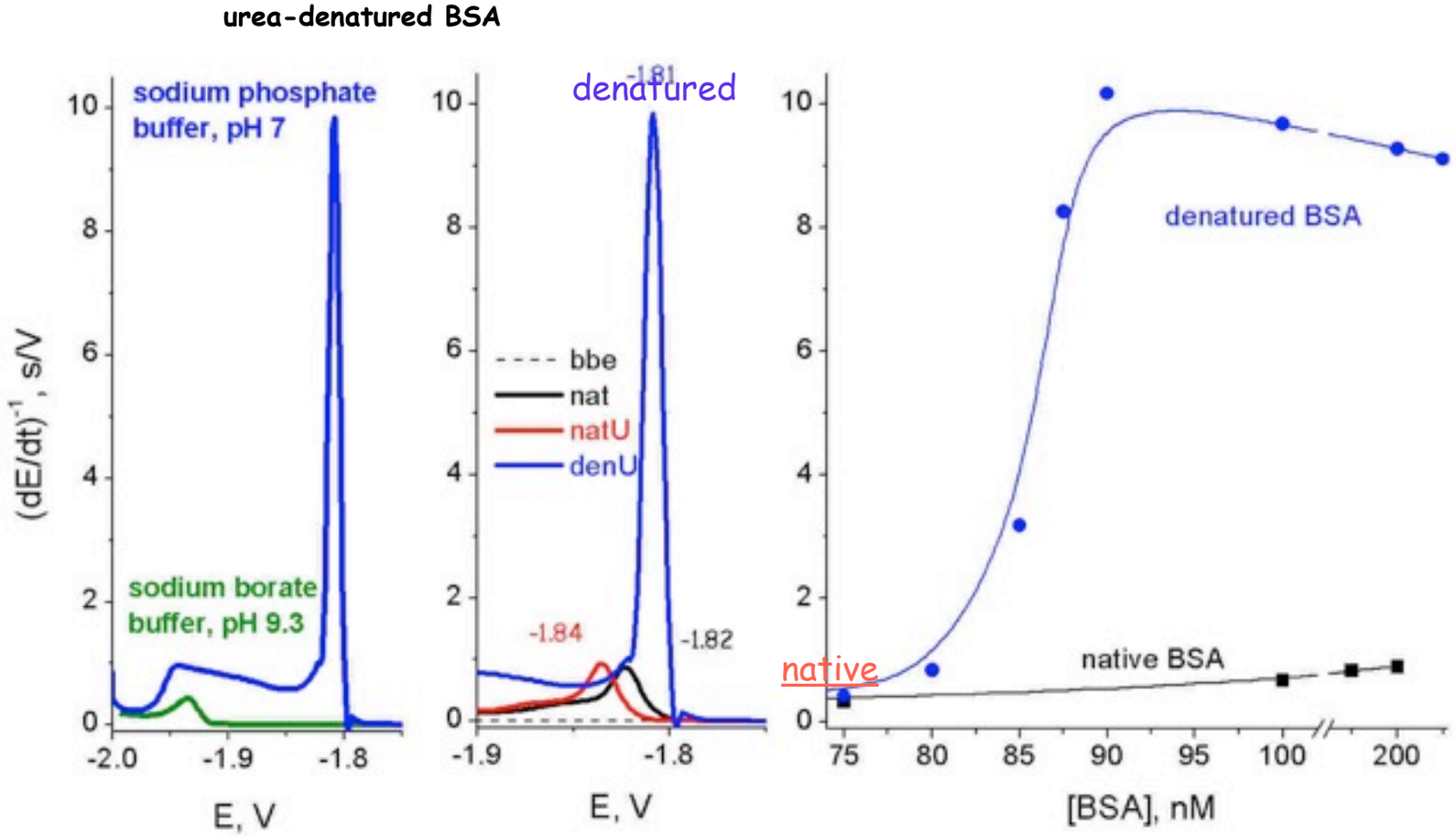
denatured

Denatured proteins produce much higher peak H than their native forms (both in alkaline and neutral media)

Previously shown measurements were performed at **alkaline pH's** (around pH 9.3)
Qualitatively similar results were obtained also with other proteins such as **human serum albumin, γ-globulin, α-globulin, concanavalin, α-crystallin, myoglobin, avidin,** etc.

I will show that **also at neutral pH** large differences between peak H heights of native and denatured BSA and other proteins can be observed. On the other hand **under certain conditions denaturation of BSA at the electrode surface may take place.**

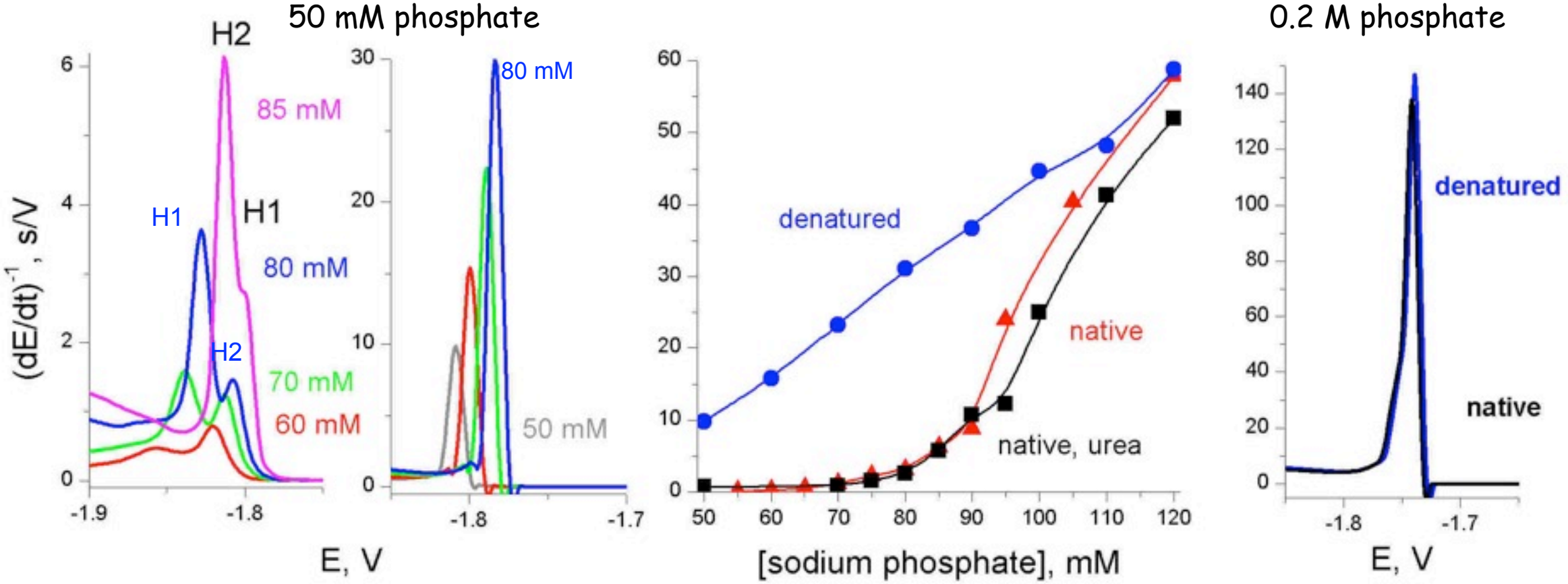
50 mM sodium phosphate, pH 7.0



Such a large difference between peaks of native and denatured BSA can be observed only at very fast potential changes as in CPS at relatively high stripping currents. Much smaller differences were found with CV at 4 V/s scan rate.

Ionic strength-dependent BSA transition at the electrode surface

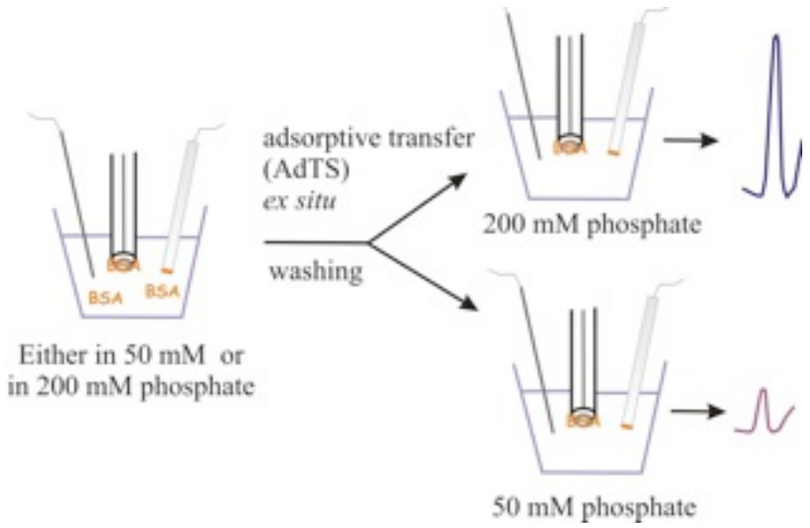
native BSA denatured BSA

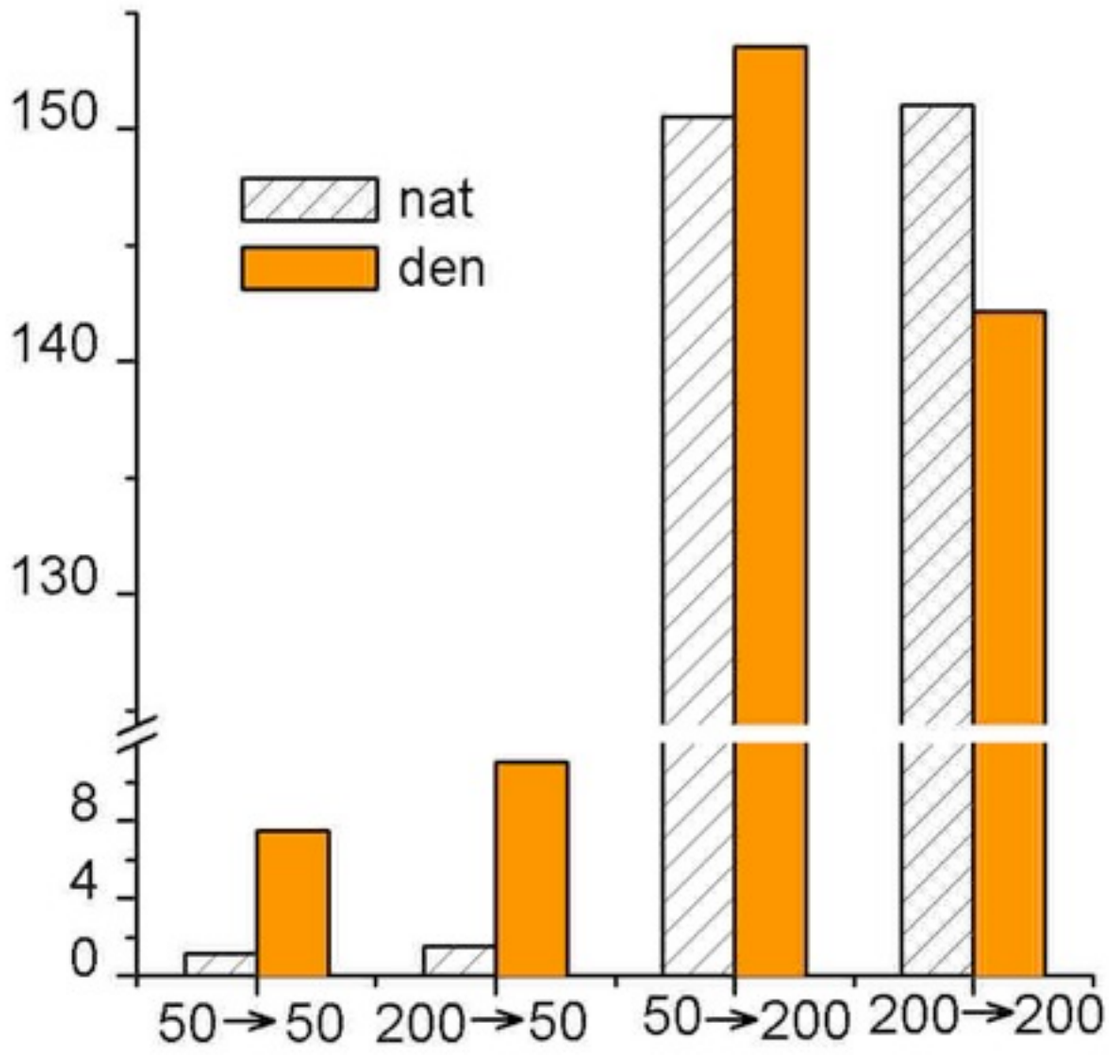


100 nM native and urea-denatured BSA in sodium phosphate (pH 7) in presence 56 mM urea, I_{str} -30 μA,, t_A 60 s, E_A -0.1 V.

Such transition is NOT observed **in solution** as indicated by the protein CD and fluorescence spectra

Is BSA denatured on HMDE in **0.2 M sodium phosphate,** pH 7 **at -0.1 V** (against SCE) ?



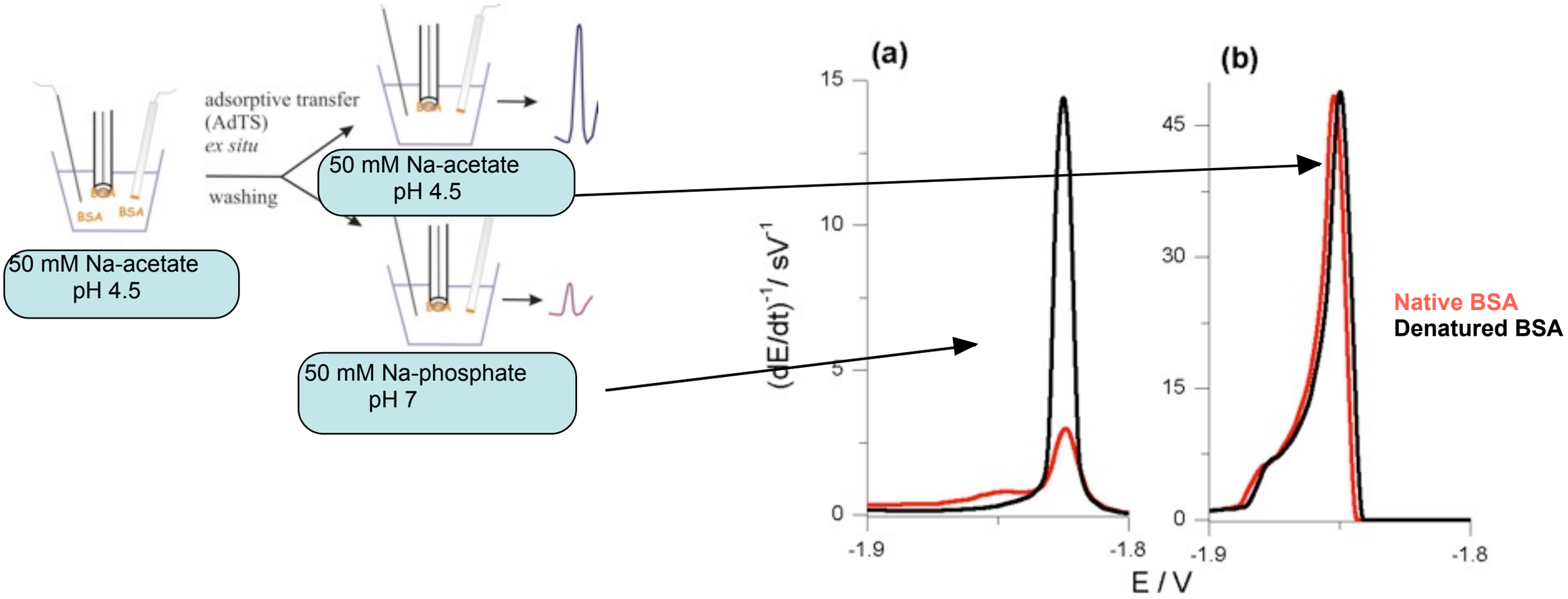


100 nM BSA was adsorbed at HMDE from 50mM/200 mM sodium phosphate pH7 (for t_A 60 s at E_A -0.1 V) in presence of 56 mM urea and then transferred either to 50 or 200 mM sodium phosphate, pH7, where the chronopotentiogram was recorded (ex situ)

Ex situ experiments indicated that **adsorption from 0.2 M sodium phosphate**, pH 7 does **not result in BSA surface denaturation**. Such denaturation does **take place**, however, **at more negative potentials.**

At neutral pH **BSA is not denatured around pzc at the Hg** electrode surface.
BSA surface denaturation can take place **at more negative potentials** and higher ionic strengths

Surface denaturation of proteins in acid media

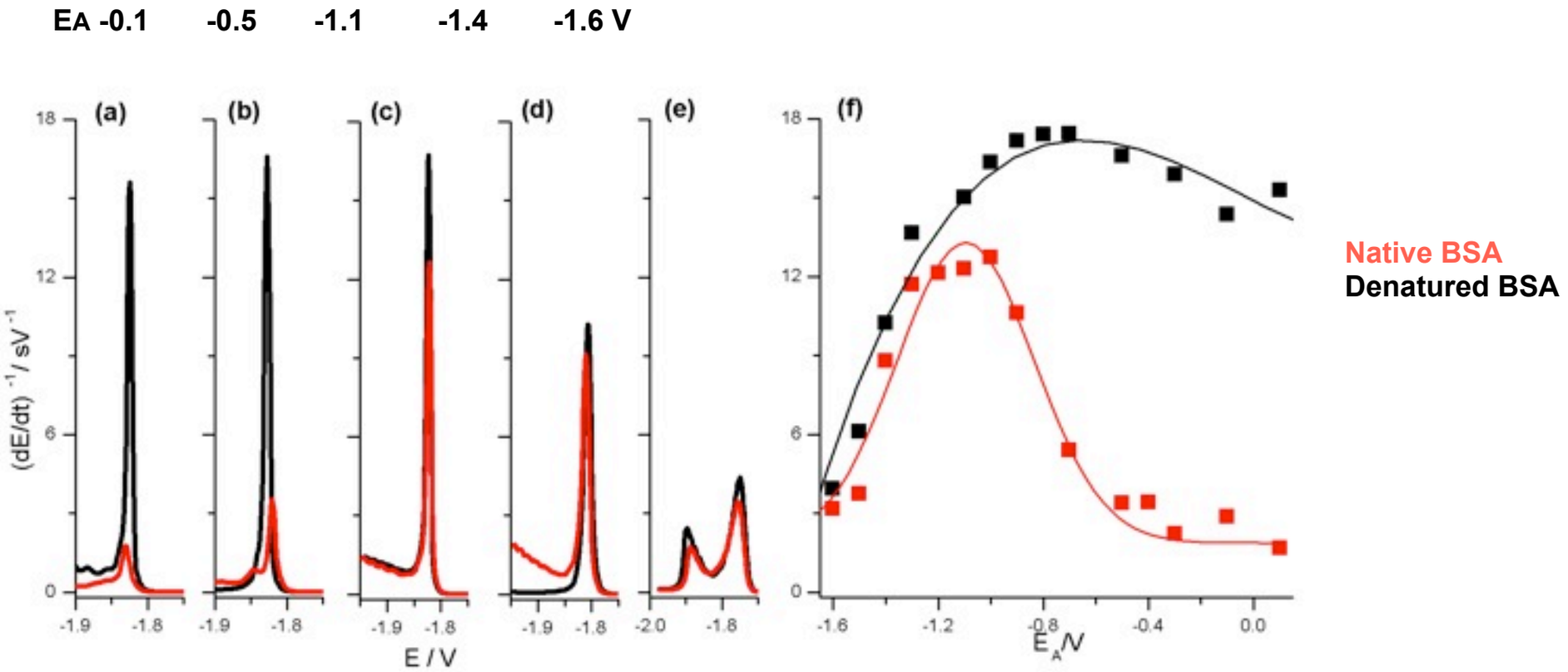


BSA in acid media is not denatured when adsorbed at -0.1 V but it undergoes surface denaturation at more negative potentials

Ex situ peak H of native (red) and denatured BSA (black) adsorbed at HMDE from 50 mM sodium acetate pH 4.5 in presence of urea 56 mM. BSA-modified electrode was transferred to **A.** 50 mM sodium phosphate buffer, pH 7 **B.** 50 mM sodium acetate, pH 4.5, where the chronopotentiogram was recorded with stripping current I_{str} -40 μA at 18.5 °C. Other details as Fig.1 C.

Palecek E., Ostatna V. (2009): Potential-dependent surface denaturation of BSA in acid media.
Analyst **134**, No. 10, 2076-2080

Dependence of peak H of native and denatured BSA on accumulation potential, E_A at pH 4.5 (ex situ as in the previous slide)



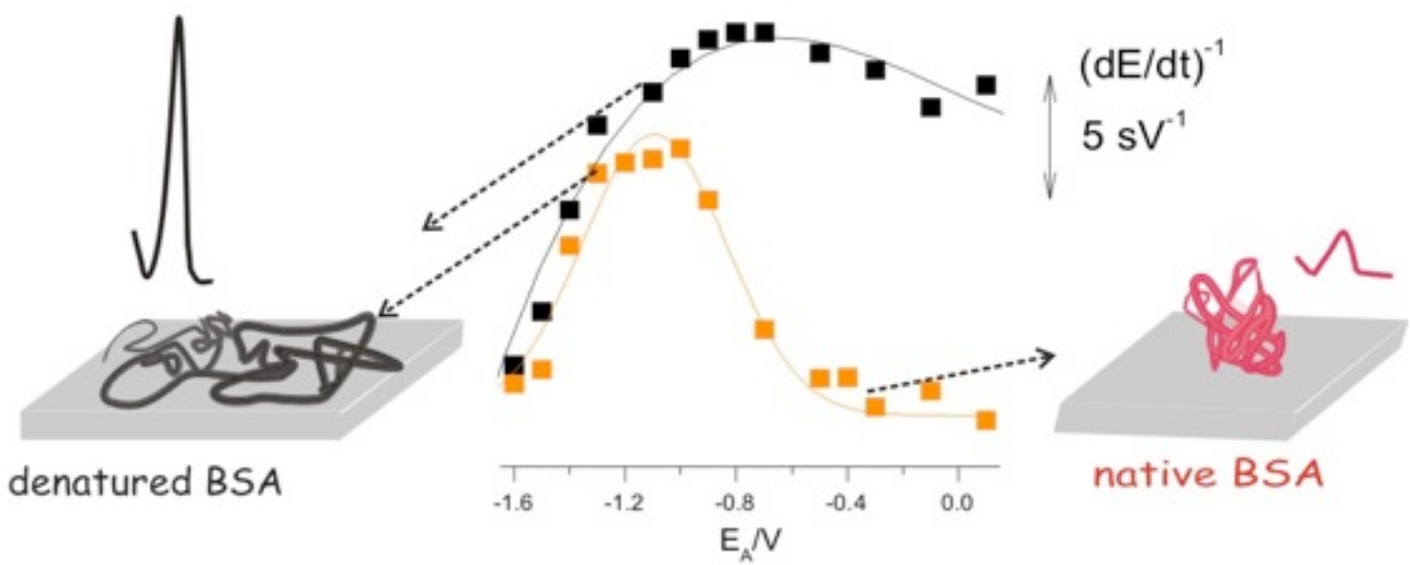
Between EA +0.1 and ~ -0.5 V surface denaturation of BSA can be neglected.
Around EA -1.1 V BSA is almost fully denatured. Compared to neutral and alkaline pH's at pH 4.5 BSA surface denaturation is much faster

100 nM native (red) and urea-denatured (black) BSA was adsorbed at HMDE for t_A 60 s at different accumulation potentials, E_A **A.** -0.1 V, **B.** -0.5 V, **C.** -1.1 V, **D.** -1.4 V and at **E.** -1.6 V from 50 mM sodium acetate pH 4.5 in presence of urea 56 mM followed by transfer of BSA modified HMDE to 50 mM sodium phosphate, pH 7. **F.** Dependence of peak H height on accumulation potential. Other details as Fig. 2B.

Denaturation of proteins at the electrode surface

At neutral pH large changes in peak H are taking place at **higher ionic strengths** with BSA immobilized at the **bare mercury electrode charged to highly negative potentials**. We **tentatively explain** these changes by **strong electric field acting on the protein** molecule tightly **bound to the mercury surface**.

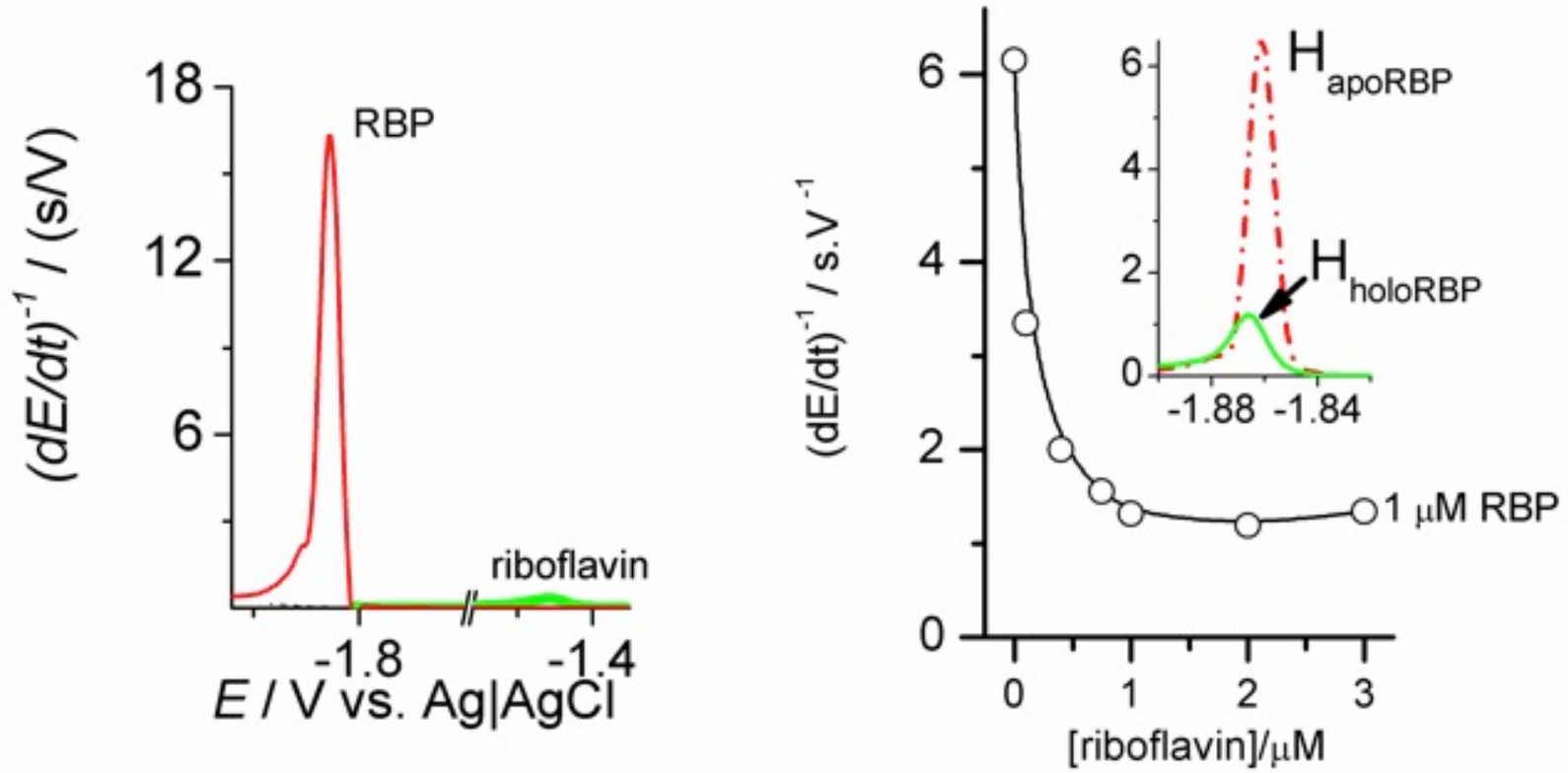
At acid pH's (e.g. pH 4.5) **native and denatured BSA produce peak H of almost the same heights**. BSA surface **denaturation** takes place at potentials negative to pzc but not at the open current potential and potentials positive to pzc.



Peak H recognizes peptide and protein redox states and specific binding of low m.w. compounds to protein molecules

Interaction of riboflavin with Riboflavin-Binding Protein

(RBP, a carrier of riboflavin, RB) plays an essential role in the embryo development.



SWV at CPE required higher concentrations of RBP and displayed almost identical oxidation peaks of apoprotein and holoprotein.

RBP produced electrocatalytic peak H, capable to discriminate between apoprotein and holoprotein forms of RBP nanomolar concentrations.

Bartosik M., Ostatna V. and Palecek E. (2009): Electrochemistry of riboflavin-binding protein and its interaction with riboflavin. Bioelectrochemistry 76, 70-75

Peptides

Angiotensin I (1296.5)
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
Angiotensin II (1046.2)

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe

Angiotensin III (931.11)

Arg-Val-Tyr-Ile-His-Pro-Phe

[Val^4]-Angiotensin III (917.08)

Arg-Val-Tyr-Val-His-Pro-Phe

Angiotensin II anti peptide (899.01)

Glu-Gly-Val-Tyr-Val-His-Pro-Val

Angiotensin IV (774.92)

Val-Tyr-Ile-His-Pro-Phe

Bradykinin (1060.2)

Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

[Met(O)^5]-Enkephalin (589.7)

Tyr-Gly-Gly-Phe-Met(O)

α_1-Mating Factor (1684.0)

Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr

Bombesin (1619.87)

Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2

SH38 (1154.2)

Asn-Arg-Cys-Ser-Gln-Gly-Ser-Cys-Trp-Asn

SS38 (1152.2)

Asn-Arg-Cys-Ser-Gln-Gly-Ser-Cys-Trp-Asn [–S-S– 3-8]

[Lys^8]-Vasopressin (1056.2)

Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH_2 [–S-S– 1-6]

Proteins

bovine serum albumin

human serum albumin

γ-globulin

α-crystalline

myoglobin

α-globulin

concanavalin A

aldolase A

lysozyme

micrococcal nuclease

RNA-ase A

azurin

cytochrom C

α-synuclein and its mutants

β-synuclein

protein p53 and its mutants

p53 core domain and its mutants

p53 C-terminal domain

insulin

thioredoxin

MutS

metallothionein

histones

avidin

streptavidin

Peptides and proteins producing peak H

all tested peptides and proteins yielded peak H

in contrast to BCR for peak H
presence of cysteine in peptides or proteins is not necessary

peptides without cysteine produced peak H only at neutral and acidic pH but not at alkaline pH

amino acid residues with labile protons such as **lysine, arginine, cysteine** are responsible for peak H

$2\ PH_{(surf)} + 2e \text{ -- } 2\ P^-_{(surf)} + H_{2(g)}$

$P^-_{(surf)} + BH_{(aq)} \text{ -- } PH_{(surf)} + B_{(aq)}$

Recently we found that **sulfated polysaccharides** such as carrageenans and dextran sulfate produce **peak H_{PS}**

S. Strmecki et al. Electrochem.Commun. (2009) in press

Palecek E., Ostatná V. (2007): Electroactivity of non-conjugated proteins and peptides. Towards electroanalysis of all proteins. Electroanalysis, 19/23, 2383-2403.

Concluding remarks

All tested peptides and **proteins** produced CPS **peak H**. Differences in peak heights and potentials were observed in different peptides and proteins. Peak H **responds sensitively to changes in protein structures**. Very fast potential changes in CPS play an important role in this analysis. **BSA is NOT denatured at potentials close to zero charge** but surface **denaturation** may occur **at negative potentials.**

Ionic strength-dependent surface denaturation of BSA at neutral pH and potential-dependent denaturation at acid pH can be observed.

Redox states in peptides and proteins (such as thioredoxin or protein p53) can be easily determined by means of peak H. The observed differences in this peak result probably from different adsorption modes of reduced and oxidized forms at the positively charged mercury surface. Similarly recognition of holo- from apo RBP forms required protein adsorption at positive potentials.

In addition to various peptides and proteins we are currently studying **two proteins important in biomedicine** by electrochemical methods:

Tumor suppressor protein p53

declared „**The Molecule of the Year" by Science magazine in 1993** perhaps the **most important protein in the development of cancer. DNA-p53 protein interactions** are very important in performing the p53 function. **Electrochemical signals of DNA and p53 protein can be utilized in studies of these interactions**

α-synuclein (Asyn)

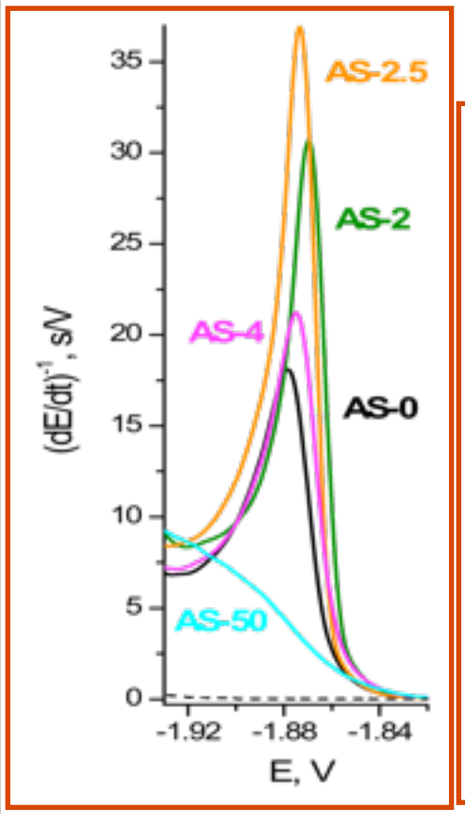
a major component of Lewy bodies associated with **Parkinson disease**. It is **natively unfolded** but undergoes **aggregation leading to fibrillar structures**, in which the protein adopts a **β-sheet secondary structure**

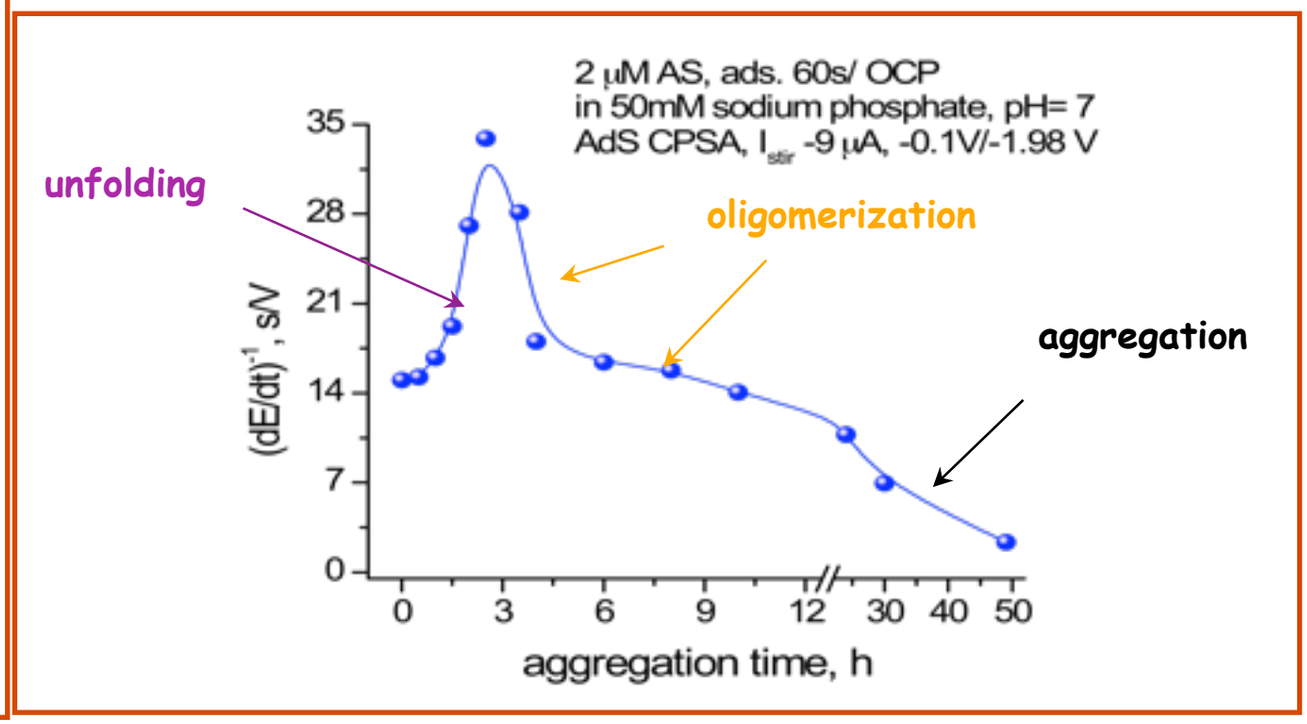
Understanding the **mechanism of aggregation** and the factors that modulate it, is important for devising **therapeutic strategies.**

The number of methods for studying the process of aggregation is limited and **electrochemistry** appears to be **suitable** for this purpose.

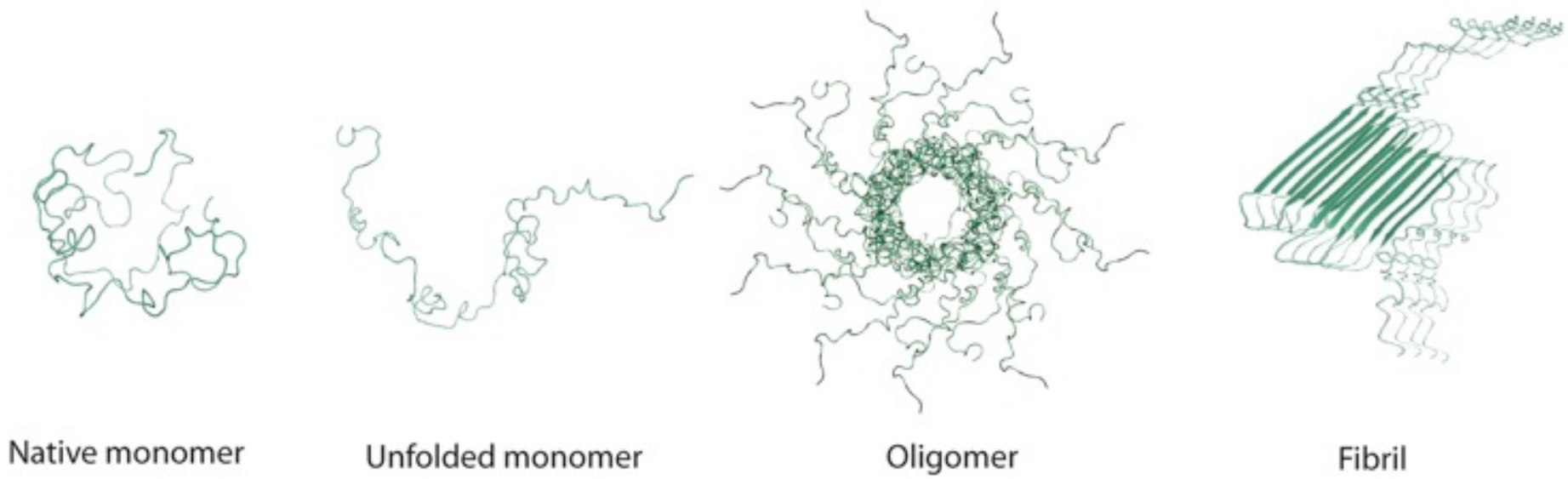
CONSTANT CURRENT CHRONOPOTENTIOMETRY of Asyn (natively unfolded protein)

Great changes in peak H not only during the AS aggregation and formation of mature fibrils but particularly during the early stages of AS incubation in vitro.





NO Cys, NO Trp in the molecule



Palecek, E.,et al. (2008): Changes in interfacial properties of α-synuclein preceding its aggregation. Analyst 133, 76-84.

Amino acid sequence of human tumor suppressor **p53 protein**

```
MEEPQSDPSV EPPLSQETFS DLWKLLPENN VLSPLPSQAM DDLMLSPDDI EQWFTEDPGP

        70          80          90          100         110         120
         |           |           |           |           |           |
DEAPRMPEAA PPVAPAPAAP TPAAPAPAPS WPLSSSVPSQ KTYQGSYGFR LGFLHSGTAK

       130         140         150         160         170         180
         |           |           |           |           |           |
SVTCTYSPAL NKMFCQLAKT CPVQLWVDST PPPGTRVRAM AIYKQSQHMT EVVRRCPHHE

       190         200         210         220         230         240
         |           |           |           |           |           |
RCSDSDGLAP PQHLIRVEGN LRVEYLDDRN TFRHSVVVPY EPPEVGSDCT TIHYNYMCNS

       250         260         270         280         290         300
         |           |           |           |           |           |
SCMGGMNRRP ILTIITLEDS SGNLLGRNSF EVRVCACPGR DRRTEEENLR KKGEPHHELP

       310         320         330         340         350         360
         |           |           |           |           |           |
PGSTKRALPN NTSSSPQPKK KPLDGEYFTL QIRGRERFEM FRELNEALEL KDAQAGKEPG

       370         380         390
         |           |           |
GSRAHSSHLK SKKGQSTSRH KKLMFKTEGP DSD
```

Amino acids which can contribute to the p53 protein electroactivity are shown as larger letters. C, cysteine; W, tryptophan; Y, tyrosine; R, arginine; K, lysine. Core domain is underlined.
N-terminus is acidic, C-terminus is strongly basic

The **N-terminal domain** (aa 1- ~100) contains a transactivation region (aa 1- 42) and a proline-rich region; this domain contains **two Trp's** (W), one of them in the transactivation region, but **no Tyr and Cys** (C) residues.

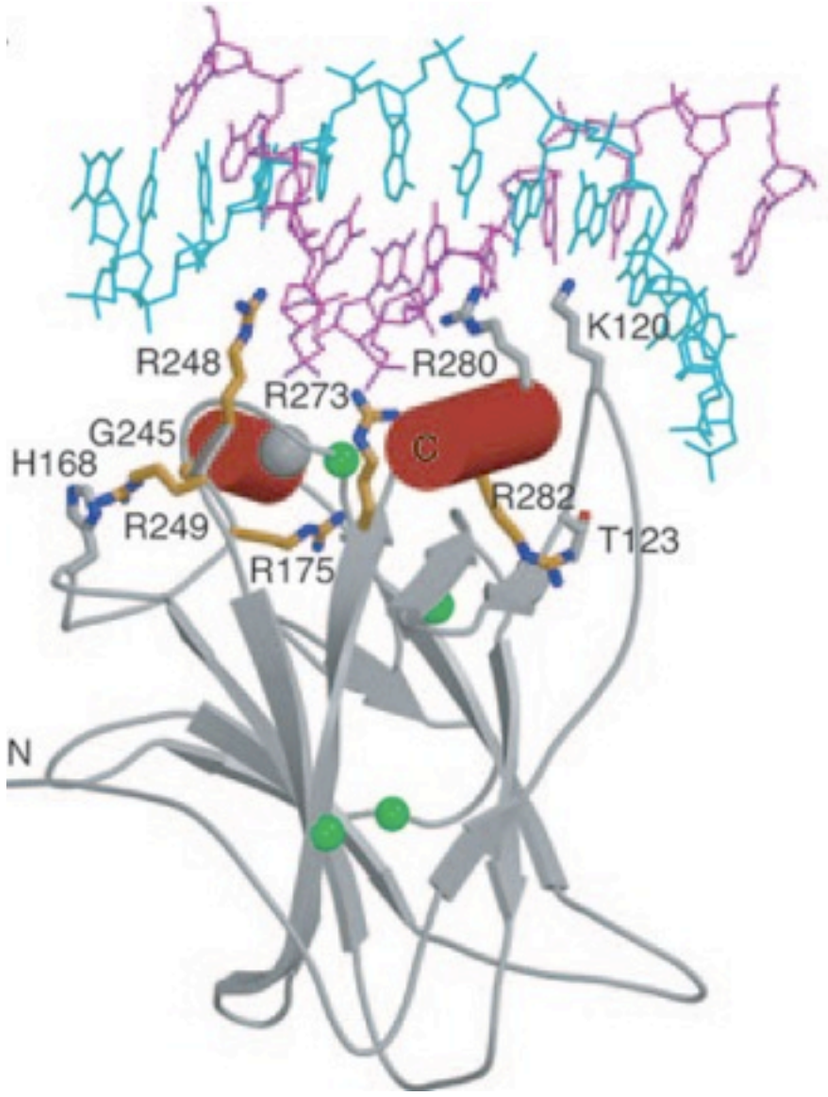
The **central (core) domain** (CD, aa ~100- ~300) is involved in sequence-specific binding to DNA
contains all Cys in p53 molecule

C-terminal domain is involved in non-specific binding to DNA and contains **no Trp and Cys and only one Tyr**

Superstable p53 T-p53C core domain

The low thermodynamic stability of wt p53 protein makes the protein difficult to study by biophysical and structural methods.

More suitable for many experimental purposes were stabilized p53 variants. Stabilization of p53 means an increase in the thermodynamic stability of the protein, derived from its folding -unfolding equilibrium.



The most stable substitutions forms superstable quadruple mutant (T-p53C) **M133L/V203A/N239Y/N268D**.

The T-p53C provides a more rigid and stable structural framework, while **maintaining the overall structural characteristics** of the wild type protein.

T-p53C was received from Prof. Alan R Fersht from MRC, Cambridge UK
p53 core domain Prof. Z. Shakked from Weizmann Institute, Rehovot, Israel

We applied the electrochemical method to study p53 protein and showed that

(i) redox states of core domain p53 can be determined and

(ii) wild type and mutant p53 produce different electrochemical signals depending of 3D structure at different temperatures

(iii) The responses of wt differed from tested mutants in a wide temperature range in a different extent. Responses of F270L and V143A around 20 °C were almost the same.

(iv) We followed the time dependence of the EDTA effect on CPSA peak H1 and H2 of superstable Tp53C (MRC), p53 CD (WIS). Tp53C displayed much slower kinetics as compared to p53 CD. Treatment of R175H with EDTA had no effect on the mutant responses.

Label-free assay of DNA-protein interactions

using double-surface technique.

Suitable for almost all proteins

Determination of point mutations by MutS protein

Paleček, E. et al. (2004). "Sensitive electrochemical determination of unlabeled MutS protein and detection of point mutation in DNA." ***Anal. Chem.*** **76**(19): 5930-5936.

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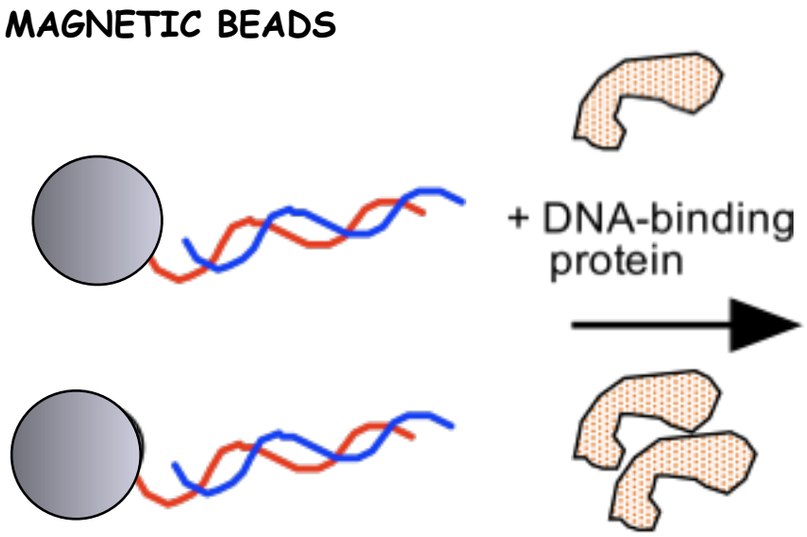

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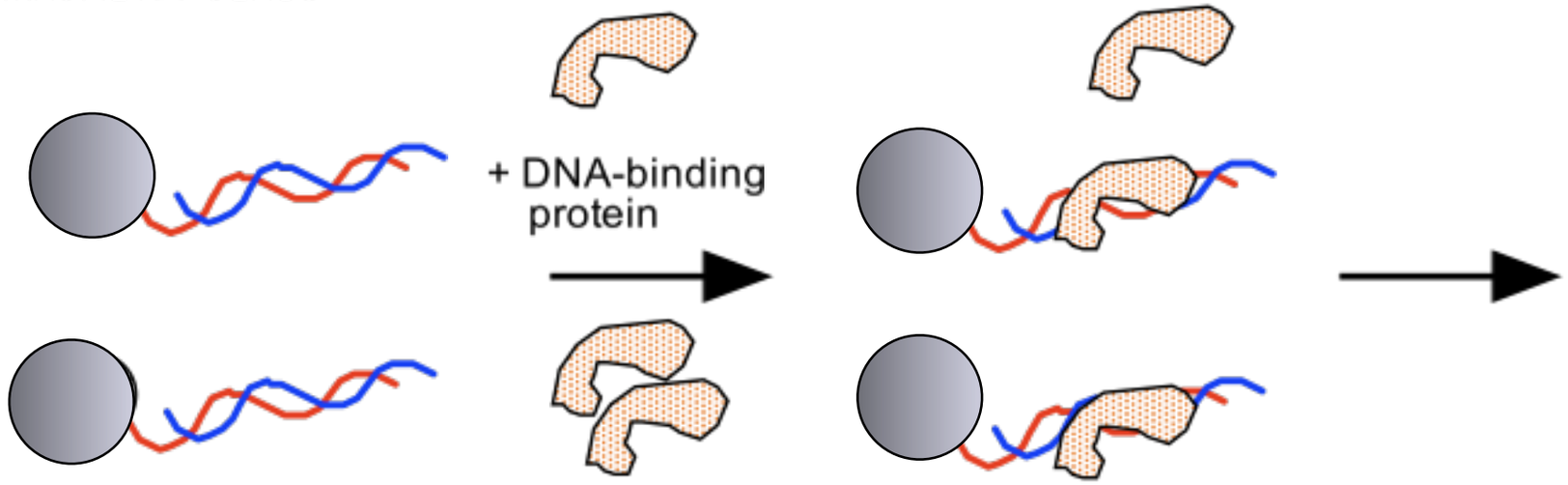

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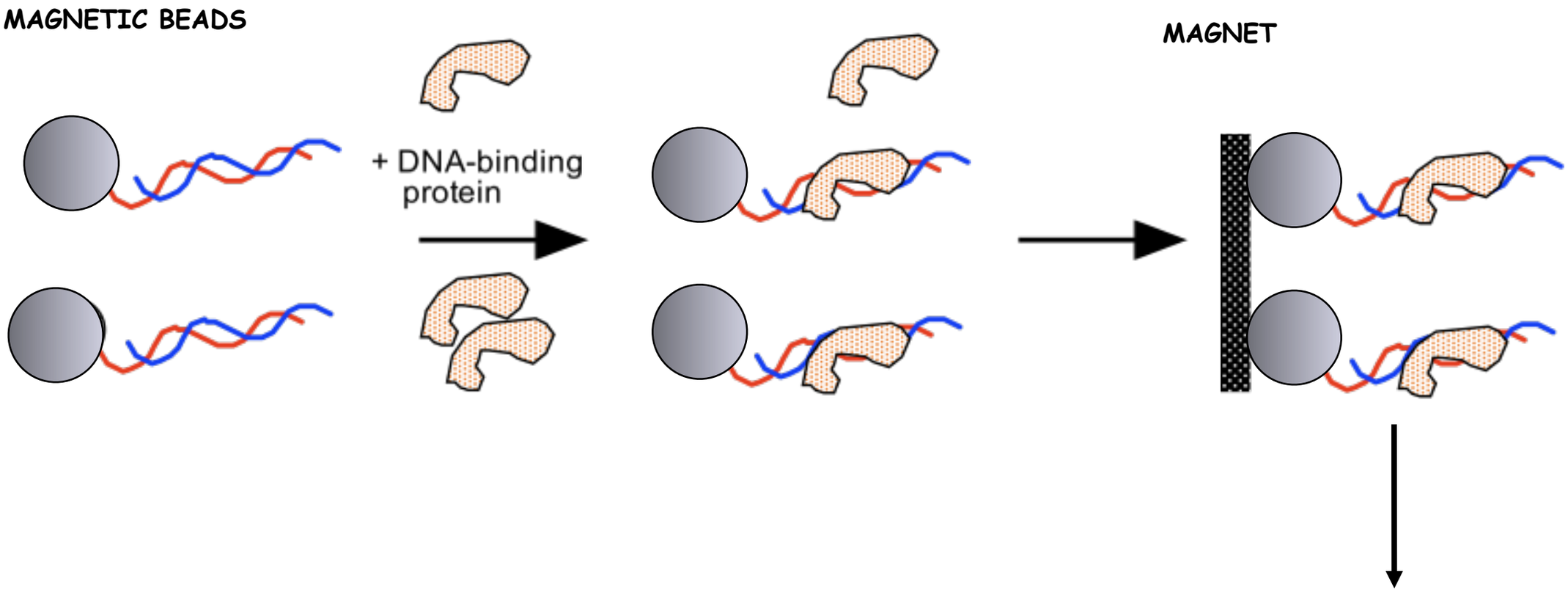

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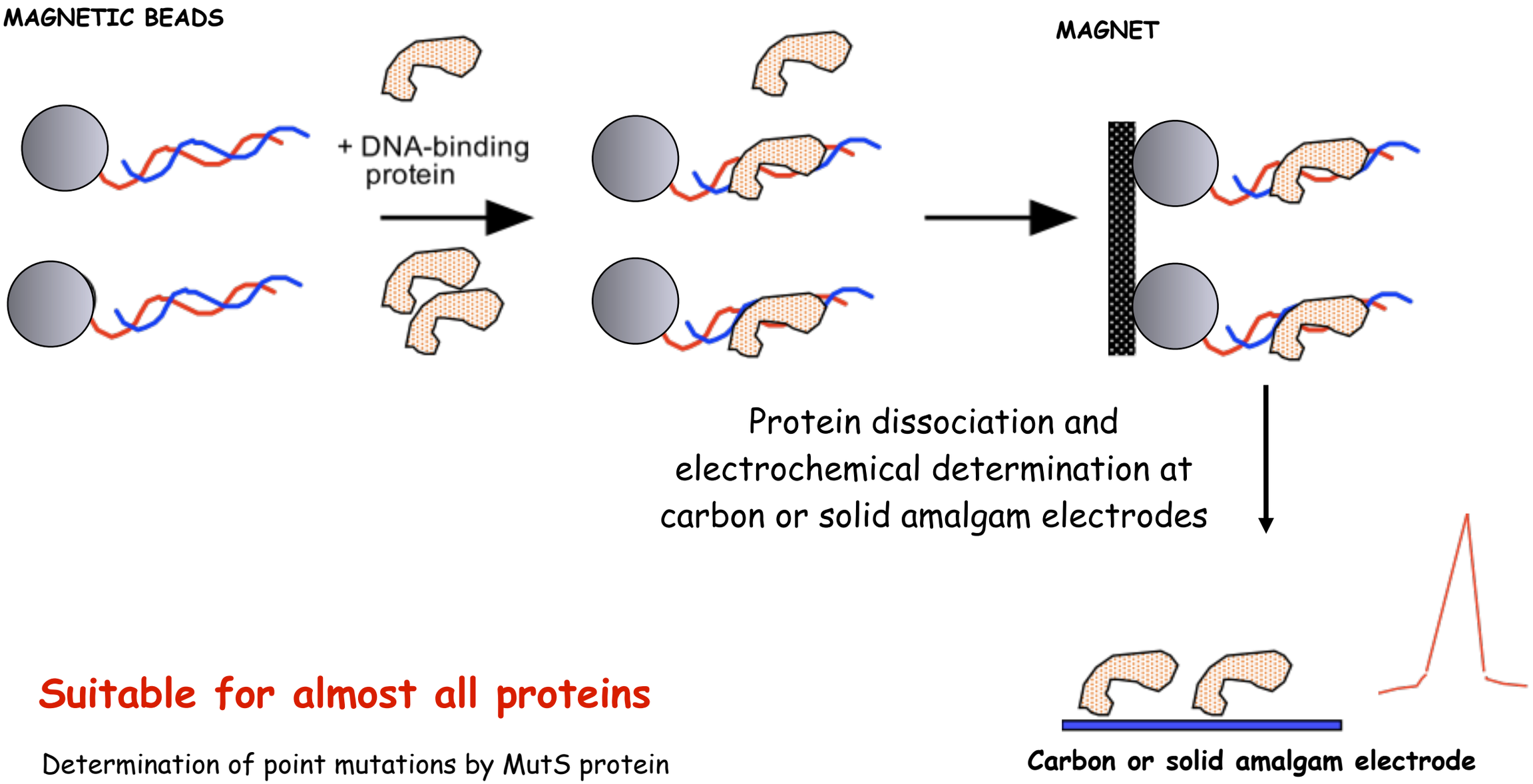
Paleček, E. et al. (2004). "Sensitive electrochemical determination of unlabeled MutS protein and detection of point mutation in DNA." ***Anal. Chem.*** **76**(19): 5930-5936.

Label-free assay of DNA-protein interactions

using double-surface technique.



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ELECTROCHEMISTRY OF BIOMACROMOLECULES

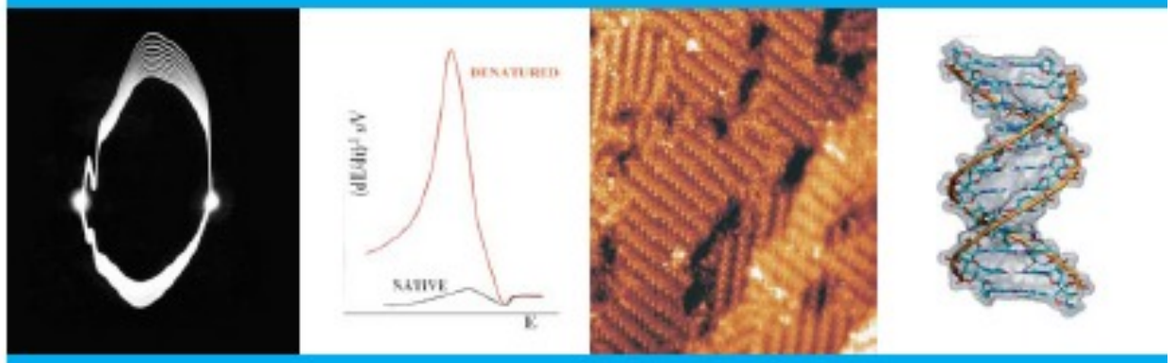
First polarographic measurements of **proteins** were done in J. Heyrovský laboratory about 80 years ago. About 30 years later electrochemistry entered the field of **nucleic acid** research, followed by application of electrochemical analysis of lipids and membranes. Until very recently electrochemical analysis of polysaccharides was limited to a few papers dealing with adsorption phenomena. Recent labeling of **polysaccharides** with Os(VI)L complexes and finding the ability of (unlabeled) sulfated polysaccharides to catalyze hydrogen evolution and produce peak H_{PS} open the door for wider use of electrochemistry in the polysaccharide research. Thus at present electrochemistry can be applied in the research of four main classes of biomacromolecules, i.e. PROTEINS, NUCLEIC ACIDS, LIPIDS AND POLYSACCHARIDES.

PERSPECTIVES IN BIOANALYSIS

VOLUME 1

ELECTROCHEMISTRY OF NUCLEIC ACIDS AND PROTEINS

Towards Electrochemical Sensors for Genomics and Proteomics



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Merry Christmas and
a Happy New Year
2010!